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Glycosaminoglycan-Dependent Isolation of Mesenchymal Chondroprogenitor Populations from Human Bone Marrow

A thesis submitted to the National University of Ireland as fulfilment of the requirement for the degree of

Doctor of Philosophy

By

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To my two best buddies and my constant companions, my dogs, Cooper and Bailey, they deserve this mention as they kept me sane for the six months that I was writing my thesis in the basement “dungeon”! My two faithful, constant companions I love you and our newer addition Daisy who has made my life brighter.
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Declaration

I declare that all the work in this thesis was performed personally. No part of this work has been submitted for consideration as part of any other degree or award.
### Abbreviations

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<tbody>
<tr>
<td>a-MSC</td>
<td>Adipose-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BML</td>
<td>Bone marrow lesions</td>
</tr>
<tr>
<td>BMMNC</td>
<td>Bone marrow mononuclear cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca++</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCM</td>
<td>Complete chondrogenic medium</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony forming unit fibroblast</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CPD</td>
<td>Cumulative population doublings</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin Sulfate</td>
</tr>
<tr>
<td>C-6-S</td>
<td>Chondroitin-6-Sulfate</td>
</tr>
<tr>
<td>C-4-S</td>
<td>Chondroitin-4-Sulfate</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>ΔCt</td>
<td>Delta threshold cycle</td>
</tr>
<tr>
<td>ΔΔCt</td>
<td>Delta-delta threshold cycle</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celsius</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMMB</td>
<td>1,9-dimethylmethylene blue</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>DPX</td>
<td>Distyrene plasticizer/xylene</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>EA</td>
<td>Early Adherent</td>
</tr>
<tr>
<td>eSP</td>
<td>Early Sub-Population</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GvHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
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</table>
$H_2O$ Water
HA Hyaluronic Acid/Hyaluronan
HA EA Hyaluronic Acid Early Adherent
HAeSP Hyaluronic Acid Early Sub-Population
HA+HA Hyaluronic Acid plus Hyaluronic Acid adherent cell supernatant
HA LA Hyaluronic Acid Late Adherent
HAISP Hyaluronic Late Sub-Population
HA+PL Hyaluronic Acid plus Plastic adherent cell supernatant
HCl Hydrochloric acid
HEPES 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
HS Heparan Sulfate
ICM Incomplete chondrogenic medium
IgG Immunoglobulin G
IgM Immunoglobulin M
IMS Industrial methylated spirit
ISCT International Society for Cell Therapy
IU International unit
KIU Kilo International unit
L Litres
LA Late Adherent
ISP Late Sub-Population
LNGFR Low-affinity nerve growth factor receptor
M Molar (moles per L)
mAb Monoclonal antibody
MACS Magnetic-activated cell sorting
mg Milligrams
Mg$^{++}$ Magnesium
µg Micrograms
µl Microlitres
µM Micromolar
µm Micron
min Minutes
ml Millilitres
mM Millimolar
mm Millimetre
MNC Mononuclear cell
MSC Mesenchymal stem cell
NaCl Sodium chloride
NEAA Non-essential amino acids
ng Nanograms
nm Nanometre
OA Osteoarthritis
PBMC Peripheral blood mononuclear cells
PBS Phosphate buffer saline
<table>
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<th>Description</th>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE/A</td>
<td>Plastic Early Adherent</td>
</tr>
<tr>
<td>PeSP</td>
<td>Plastic Early Sub-Population</td>
</tr>
<tr>
<td>PD</td>
<td>Population doublings</td>
</tr>
<tr>
<td>PL</td>
<td>Plastic</td>
</tr>
<tr>
<td>P LA</td>
<td>Plastic Late Adherent</td>
</tr>
<tr>
<td>PISP</td>
<td>Plastic Late Sub-Population</td>
</tr>
<tr>
<td>PL+HA</td>
<td>Plastic plus hyaluronic acid adherent cell supernatant</td>
</tr>
<tr>
<td>PL+PL</td>
<td>Plastic plus plastic adherent cell population</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue Culture Plastic</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor bet</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>x g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>X-ray</td>
<td>X-radiation</td>
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Abstract

Osteoarthritis (OA) is a chronic disease of joints characterised by progressive destruction of articular cartilage resulting in painful, limited joint movement. Cartilage has a limited ability to self-repair due to low chondrocyte motility and proliferative rates, and is further complicated by the absence of blood vessels for recruitment of circulating cells. Current clinical therapies do not result in full regeneration of healthy cartilage tissue. The long-term success of cartilage repair will therefore depend on regenerative methodologies resulting in the restoration of articular cartilage that closely duplicates the native tissue. For cell-based therapies, the optimal cell source must be readily accessible with easily isolated, abundant cells capable of collagen type II and sulfated proteoglycan production in appropriate proportions. Although a cell source with these therapeutic properties remains elusive, mesenchymal stem cells (MSCs) show promise of reproducing the structural or biomechanical properties of healthy articular cartilage. Current knowledge of and selection techniques for chondroprogenitors within the MSC population are relatively limited. This study focuses on methods for their isolation and activation.

As cartilage is a tissue composed primarily of extracellular matrix (ECM) surrounding chondrocytes, it was hypothesised that there is a sub-population of progenitor cells in bone marrow that are primed towards the chondrogenic pathway with pre-requisite receptors for extracellular matrix (ECM) molecules. Consequently, chondroprogenitors could be isolated from bone marrow via their specific adhesion to cartilaginous ECM proteins. In this study hyaluronan (HA) and chondroitin-6-sulfate (CS) were used to select cells directly from bone marrow by coating tissue culture plastic or by adding in solution to unprocessed marrow. Various methods were undertaken to isolate this putative population of chondroprogenitors such as isolating the early adherent (EA) and late adherent (LA) cells and the sub-populations present as slow adherent cells in the EA and LA marrow fractions. Extracellular matrix-mediated isolation of cells, specifically the
exposure of MSCs to a specific ECM molecule adhered to tissue culture plastic and subsequent re-plating onto non-coated flasks resulted in a 9-fold higher chondrogenic ability compared to the traditionally isolated plastic adhered cells. These ECM isolated cells retained their tri-lineage potential but the increase in differentiation potential was a chondrogenic phenomenon only. Further analysis suggested that this was not a specific selection of chondroprogenitors but an activation of a chondro-specific pathway within the ECM isolated MSCs. This study has not only elucidated a process enabling the isolation of a highly chondrogenic population of cells but also a process of MSC isolation from marrow that enables the retrieval of a higher yield of cells than is typically isolated using traditional methods.
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4.10 Chondrogenic differentiation in marrow conditioned media treated MSCs

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5.1 Schematic for current state of the art in MSC isolation
**Chapter 1**

Introduction
**Introduction**

**1.1. Regenerative Medicine**

Regenerative medicine refers to the process of replacing or regenerating human cells, tissues or organs to restore normal function. Research in regenerative medicine has come very far since the 1980s. It has gone from experimentation in the laboratory to improving the lives of hundreds of thousands of people who have been treated successfully with cell and tissue-engineered therapies (Mason and Dunnill, 2008). Today, scientists and engineers are trying to create a consistent and sustainable method of regenerating damaged tissues associated with many intractable diseases and injuries. Osteoarthritis (OA) is one of these diseases that requires long term regeneration of damaged joint tissues to enable patients to regain an acceptable quality of life. OA is a chronic disease of the joints characterized by progressive destruction of articular cartilage and ultimately damage to the total joint resulting in painful and limited movement (Goldring and Goldring, 2007).

Although the ultimate option for OA is total joint replacement, several procedures such as debridement, marrow stimulation techniques (abrasion chondroplasty, microfracture, drilling), osteochondral allograft, osteochondral autograft and autologous chondrocyte implantation have been used to promote cartilage repair and prevent or impede OA progression (Browne et al., 2000; Mandelbaum et al., 1998; Sgalione et al., 2002; Fujita et al., 2012; Donnenwerth and Roukis, 2012; Brittberg et al., 1994; Behrens et al., 2005). These treatments have significant limitations such as donor site morbidity, repair cell de-differentiation, restricted cellular life span upon implantation, spontaneous osteonecrosis and poor differentiation potential of cells derived from OA patients. The invasive nature of joint replacement is also a major disadvantage (Benya et al., 1978; Diaz-Romero et al., 2005; Brittberg et al., 2003; Guerit et al., 2012; Von Keudell et al., 2011; Minas et al., 2009; Tallheden et al., 2005). These issues have led to the requirement for new cell sources for cartilage repair. Regenerative biomedical approaches and
clinical therapies can involve the use of stem cells (Riazi et al., 2009). Stem cells have enormous regenerative capacity and adult stem cells can be isolated from many of the body’s tissues leading to safe and more ethical forms of treatment (McLaren et al., 2001).

1.2 Stem Cells
1.2.1 Stem Cells

At the blastocyst stage, stem cells exist within the embryo proper, in the inner cell mass. These embryonic stem cells (ES) have the capacity for prolonged and unlimited self-renewal and can produce highly differentiated descendants. In vitro studies using ES cells for instance have shown that these cells can be propagated indefinitely in an undifferentiated state but can be differentiated to all mature cell types when provided with appropriate signals (Thomson et al., 1998; Amit et al., 2000; Daadi and Steinberg, 2009; Pal, 2009; Lu et al., 2009). Stem cells provide a theoretically inexhaustible supply of cells that give rise to some or all body tissues, these types of stem cells are referred to as pluripotent cells. They have the ability to maintain themselves throughout the entire lifetime of an organism (Barry, 2003).

Current research is based on promoting differentiation of stem cells to whichever lineage is required, the derivation of a highly purified non-carcinogenic population and the implantation in a form that will replace or improve the function of diseased or degenerated tissues (Odorico et al., 2001; Daadi and Steinberg, 2009; Pal, 2009).

Stem cells were first described over 140 years ago with the term appearing in scientific literature as early as 1868 in the works of the eminent German biologist Ernst Haeckel (Haeckel, 1879). Haeckel was a major supporter of Darwin’s theory of evolution, so he drew phylogenetic trees to represent the evolution of organisms by descent from common ancestors. He called these trees “Stammbaume” (German for family trees or “stem trees’”). Haeckel used the term “Stammzelle” (stem cell) to describe the preliminary unicellular organism which he presumed all multicellular organisms evolved from (Heackel, 1879). From this beginning
scientists went about trying to understand how these cells become the different organs and tissues in the body (Becker et al., 1963; McCulloch et al., 1965; Ramalho-Santos and Willenbring, 2007). Culture of mouse ES cells was first reported by Evans and Kaufman in 1981. Bongso et al then described the culture of human ES cells (Bongso et al., 1994, Evans and Kaufman, 1981). Special interest and excitement began building in this area when, in 1998, Thompson and colleagues succeeded in isolating, culturing and expanding human embryonic stem cells (Thomson et al., 1998). Thomson’s work offered an insight into the process of embryonic development. ES cells are isolated from the early embryo, specifically from the inner cell mass of blastocysts, and are an attractive therapeutic cell type due to their pluripotency (Thomson et al., 1998). The idea of being able to grow and manipulate these stem cells caused much excitement at this time because they had a putative intrinsic capacity to self-renew and differentiate into all functional cell-types and thus, re-constitute tissues that are diseased or damaged.

1.2.2 Embryogenesis

During the embryo’s early development the three germ layers, the endoderm, the mesoderm and the ectoderm differentiate down specific paths to form all the cell types in the body. Derivatives of the mesoderm include bone, muscle and cartilage cells, which are the building blocks of the skeletal system. The mesoderm develops from the ectoderm in a process known as the first epiblast-mesenchyme transition. The mesoderm is the founder of the mesenchymal cells which will later on in development be found in the connective tissues (Takashima et al., 2007; Pittenger et al., 1999; Prockop et al., 1997). Therefore, between the originating stem cells and their terminally differentiated progeny, there are populations of intermediate committed progenitors with more limited proliferative capacity and with more restricted differentiation potential. These multipotent stem cells can be isolated from different sources in the adult body. One such type is the mesenchymal stem cell (MSC).
These adult stem cells are considered to be developmentally committed to produce specific cell lineages only. These lineages would namely be those from the tissue in which the stem cells reside; for example, epithelium stem cells form epithelial cells or cardiac stem cells form cardiac tissue (Alonso and Fuchs, 2003; Beltrami et al., 2003). Besides the stem cells producing the tissue in which they reside in, they also gave rise to a set of non-related progenitors. Petersen et al. demonstrated that bone marrow can be a source of hepatic oval cells (Petersen et al., 1999). Neural stem cells have originated cells that are found in the adult brain as shown by Morrison et al. and McKay et al. (Morrison et al., 1997; McKay et al., 1997). However, these have also been shown to produce early and lineage committed hematopoietic progenitors (Bjornson et al., 1999). Biologically, there may be a need for more precursors upon the onset of injury or disease, thus adult organisms require the ability to recruit uncommitted progenitors from other tissue sources in such cases of tissue growth and repair. This was proven to be the case during muscle repair when mesenchymal cells in the bone marrow were shown to migrate to skeletal muscle (Ferrari et al., 1998). Adult stem cells are extremely malleable and show a high degree of plasticity. They are found and have been isolated from a number of different tissues (Friedenstein et al., 1966; De Ugarte et al., 2000; Arai et al., 2002; English et al., 2007; Pei et al., 2008; Maxon et al., 2012). These cells have generated huge amounts of interest because they have the potential to be very useful in regenerative medicine and tissue engineering. Clinical studies have shown dramatic examples that demonstrate the therapeutic value of adult stem cells, MSCs in particular.

1.2.3 Mesogenesis

MSCs (also referred to as mesenchymal progenitor cells) are mobile cells which have migrated from their original stem cell niche in the embryo and, thus, belong to the new transient group of more specific cells (Pittenger et al., 1999; Dennis et al., 1999; Muraglia et al., 2000). Cells from the bone marrow that demonstrated the ability to stimulate bone formation both in vivo and in vitro were identified in early
work (Friedenstein et al., 1966). These cells were first identified as precursors from bone marrow which demonstrated plastic adherence and formed fibroblast-like colony forming units (CFU-F) (Friedenstein et al., 1970; Owen and Friedenstein, 1988). These early scientists referred to the cells as marrow stromal cells or mesenchymal stromal cells. Subsequent work carried out by Caplan and colleagues demonstrated that these cells exhibited stemness properties and could give rise to various tissues of the mesenchymal lineage (Caplan, 1991); thus these progenitor cells were referred to as mesenchymal stem cells. For the purposes of this thesis, MSCs refers to mesenchymal stem cells.

Following on from the early work carried out describing MSCs in marrow as plastic adherent cells with colony forming ability and tri-lineage differentiation ability (Pittenger et al., 1999; Muraglia et al., 2000), it soon became apparent that cells with characteristics of MSCs are readily available from a variety of adult mesenchymal tissues such as synovium (De Bari et al., 2001), periosteum (Nakahara et al., 1990; Sakaguchi et al., 2005), skeletal muscle (Cao et al., 2001), adipose tissue (Zuk et al., 2002), trabecular bone (Sakaguchi et al., 2004) and umbilical cord blood (Lee et al., 2004). These cells have extensive proliferation potential and are expanded easily without loss of their multilineage differentiation potential within several passages. The intermediate processes are outlined in Figure 1.1 below, showing how during embryonic development, the mesodermal layer harbours multipotent progenitor cells that give rise to bone, cartilage, muscle and other tissue of the mesenchyme. The fact that stem cells can be isolated from adult tissues enabled scientists to avoid the ethical issues involved with using the pluripotent stem cells from embryos associated with the inevitable destruction of the embryo when utilising the embryonic stem cells (McLaren et al., 2001).
Figure 1.1: The mesengenic process. This diagram describes how MSCs can be differentiated into several mesenchymal lineages; bone, cartilage, muscle, marrow, tendon/ligament, adipose and connective tissue with potential intermediates for each lineage (Singer and Caplan, 2011).

1.2.4. MSC Characterisation

As described above, the term MSC has been generally accepted to describe the heterogeneous, fibroblast-like, plastic adherent cells with multi-differentiative capacity that can be isolated from the bone marrow, adipose or other tissues. However, it has been shown that the biologic properties of some of the unfractionated population of cells do not all meet the accepted criteria for typical stem cell activity, or the “stemness” of the unfractionated cell population is lacking (Horwitz et al., 2000). Studies carried out by Battula et al confirmed the heterogeneity of individual MSC clones which have been detected by other groups and show, that even narrowly defined, rare MSC populations are highly heterogeneous with respect to their phenotype, their proliferation capacity, and
their differentiation potential (Battula et al., 2009). The isolation of a more homogenous population of cells would benefit therapies using the application of MSCs for specific tissue repair. It has been proposed by Horwitz and Dominici on behalf of the International Society for Cellular Therapy (ISCT) that the term MSC be reserved for a subset of mesenchymal cells that demonstrate stem cell activity by clearly defined and stated criteria; human MSCs must adhere to tissue culture plastic, express CD105, CD73 and CD90 and not CD34, CD45, HLADR, CD14 or CD11b, CD19 or CD79α and be able to differentiate into osteoblasts, adipocytes and chondrocytes under standard *in vitro* differentiating conditions (Horwitz et al., 2005; Dominici et al., 2006). Much of the current data are insufficient to characterise unfractionated plastic-adherent marrow cells as stem cells which explains the requirement for a more specific marker or method to isolate an homogenous population of marrow cells that can comfortably be classified as MSCs.

Adult progenitor cells have been described *in vivo* and *in vitro* as cells that reside in a niche adjacent to mature, differentiated cells (Barry et al., 2001). Progenitors have been successfully retrieved from several tissue sources, including adipose, synovium, synovial fluid, perichondrium and bone marrow (Arai et al., 2002; English et al., 2007; De Ugarte et al., 2000; Friedenstein et al., 1966). Depending on the tissue source from which they are isolated, progenitors harbour distinct differentiation potential and their frequencies are variable.

There is evidence for the presence of committed verses non-committed MSCs in bone marrow derived cultures. Cells in the quiescent condition have been shown to represent a population of non-committed MSCs; this is because they do not express the osteogenic and adipogenic commitment transcription factors, Cbfa-1 and PPAR-γ2, respectively. In turn, after exposure to fetal bovine serum, these same quiescent cells give rise to committed precursors that grow quickly and are capable of terminal differentiation (Pittenger et al., 1999).
Within the hierarchy of stem cells, several classes of committed progenitors have been found to be present in bone marrow cultures. Muraglia et al., have used non-immortalized cell clones to investigate the properties of committed progenitors. Upon assessment of their differentiation potential, it was found that 30% of clones exhibited a tri-lineage potential (osteo/chondro/adipo), the other 70% exhibited either bi-lineage potential (osteo/chondro) or were purely osteogenic. There were no clones detected that had osteo/adipo or chondro/adipogenic potential only. Also, there were no clones discovered at this time that were pure chondrogenic or adipogenic lines (Muraglia et al., 2000). More recently however, further studies have identified a sub-population of MSCs that retain only chondrogenic potential (Russell et al., 2010), further described in section 1.4.

There are gaps in our knowledge of stem cells that need to be filled in order to take advantage of their full potential. There is a need to know more about the intrinsic controls of stem cells and what directs them in particular differentiation pathways. The micro-environment where the cells normally reside influences these intrinsic regulators (Watt and Hogan, 2000). There has been great interest and hope in the regenerative capacity of adult stem/progenitor cells, particularly the progenitor cells, as these could be used in the treatment of injury and disease in many specific tissues. Take for example, a current focus is in the application of chondroprogenitors, cells that are specifically pre-disposed to differentiate into mature chondrocytes, to repair articular lesions in articular cartilage and subsequently inhibit the onset of OA (Jones et al., 2008).

1.3 Articular Cartilage

There are several types of cartilage tissue, namely fibrocartilage which is found in the semilunar meniscus of the knee, elastic cartilage, found in the external ear and articular cartilage of the synovial joint (Eyre et al., 1975). The main role and function of articular cartilage which is also referred to as hyaline cartilage, is to provide a low friction surface capable of withstanding a load so as to protect the
underlying bone from pressure and stresses of load bearing. Cartilage has a low metabolic activity. It is an a neural, a vascular and a lymphatic tissue giving it a limited capacity for self-renewal or repair. Consequently, its healing process is slow and the repair tissue lacks the mechanical and physical properties necessary for fully functional cartilage (Cancedda et al., 2003).

Hyaline cartilage is a heterogeneous structure populated solely with chondrocytes (Figure 1.2). These chondrocytes exist as single cells which are contained within a dense matrix composed of collagens and proteoglycans (Buckwalter et al., 2005). This highly organised structure has an extremely important role in cartilage and is termed the extracellular matrix (ECM). Hyaline cartilage is uniquely composed of a particular type of ECM that is made up of 40-50% collagens and 20-25% proteoglycans (Cremer et al., 1998). Collagen provides most of the structural integrity of the tissue. Collagen II is the most abundant type (>90%), with types VI, X, and XI present in much smaller quantities. Aggrecan is the major proteoglycan in cartilage which fills the interstitial spaces between the collagen fibrils. Other proteoglycans present include decorin, fibromodulin and biglycan (Hildebrand et al., 1994). An important function of the proteoglycans is water retention within the cartilage to prevent dehydration. The cartilage ultra-structure is composed of up to 65-80% water, which is important for shock absorption. It is important to maintain optimal hydration as, through differering mechanisms, both dehydration and swelling can induce initial surface rupture of mildly degenerated articular cartilage; therefore, maintenance of hydration is important (Fick and Espino, 2012). Proteoglycans prevent this by their hydrophilic nature (Ulrich-Vinther et al., 2003).

There is a rapid loss of glycosaminoglycan (GAG) from articular cartilage soon after tissue damage, as demonstrated by DiMiccio et al. (2004). When there are full depth chondral defects, widespread damage to the collagen network occurs along with chondrocyte death. Once matrix disruption occurs, the homeostatic capacity of chondrocytes is exceeded and the result is the permanent inability of articular cartilage to perform its primary function (DiMiccio et al., 2004).
Figure 1.2: (A) Hyaline cartilage is an avascular, aneural and alymphatic compact tissue with copious amounts of extracellular matrix (1) with collagenous microfibrils. Chondrocytes are present in the lacunas (2). The surface of hyaline cartilage is surrounded by perichondrium (3). (B) Schematic of hyaline cartilage outlining the various layers or zones within the cartilage and its components (Danisovic et al., 2012).

It is commonly assumed that this occurs in superficial chondral lesions due to initial collagen damage. This leads to a loss in the retention of GAG. Cartilage stiffness decreases upon the loss of proteoglycans and surface integrity. This results in impairment of the load carrying capacity due to alterations in interstitial fluid dynamics (Buckwalter et al., 1994). As previously mentioned, the low metabolic nature of mature cartilage and the difficulty in repairing collagen damage, together with mechanical wear ultimately leads to chronic and progressive joint disease. The majority of large defects do not elicit a reparative response leading to the long-term prognosis of OA (Gelber et al., 2000).
1.3.1 Osteoarthritis

The term “arthritis” literally means inflammation of the synovial joint, and it encompasses a group of more than 200 diseases. OA is a chronic disease of the joints characterized by progressive destruction of articular cartilage, intra-articular inflammation with synovitis, changes in peri-articular and subchondral bone, and ultimately damage to the total joint resulting in painful, limited joint movement (Rizkalla et al., 1992; Hollander et al., 1995; Blanco et al., 1998; Buckwater and Mankin, 1998; Goldring and Goldring, 2007; Goldring and Otero, 2011; Berenbaum, 2012). Figure 1.3 shows a schematic of the main structures of a healthy and degenerated joint in osteoarthritis. OA leads to functional limitation and a reduced quality of life for the patients affected and it is the most common form of arthritis by far (Bitton, 2009). Worldwide, it is one of the leading causes of pain and disability. Prevalence is expected to rise dramatically over the coming 20 years with the increasingly aged population (Bergman et al, 2007). OA can occur in any of the synovial joints; however, the peripheral sites are the most commonly affected; the hips, knees and small hand joints (Kuettner and Cole, 2005). OA is a dynamic process which is metabolically active and involves all the joint tissues, the cartilage, the bone, synovium, ligaments and muscle. It causes softening of the articular cartilage accompanied by loss of elasticity which increases its susceptibility to damage. The pathological changes that occur are localised loss of articular or hyaline cartilage and remodelling of the adjacent bone. As articular cartilage deteriorates, there is a loss of lubricating function within the joint causing the bones to rub off one another. This causes bone spur or osteophyte formation at the margins causing more pain (Aigner et al., 2006). Cartilage pieces may become detached within the joint leading to further inflammation, structural damage and pain. Tendons and ligaments stretch as articular cartilage weakens causing additional discomfort (Broussard, 2005; Goldring and Goldring, 2007).
Figure 1.3: Schematic representation of the main structures of a healthy (left side) and degenerated (right side) joint in osteoarthritis. The normal side shows adequate space and cushioning between the bones and the OA side shows diminished articular cartilage, causing pain from friction between the bones. Bone (subchondral) is sclerotic, the joint capsule thickened and the synovial membrane activated (Aigner et al., 2006).

As the condition worsens, loss of cartilage and the presence of more and more osteophytes can cause the joint to become misshapen which eventually results in deformity. At this advanced stage, a full joint replacement is often the only viable therapeutic option. OA at particular joint sites demonstrates a consistent age-related increase in prevalence (Bergman, 2007). With the aging population that we have now, the upcoming problems are evident. It is estimated that worldwide 9.6% of men and 18.0% of women aged ≥60 years have symptomatic OA, creating an imperative for the timely development of effective treatments for the disease (Woolf et al., 2012; Murray and Lopez, 1996). Another issue arising in society is obesity and poor diet which increases the incidence of OA and progression from increased pressure on the load-bearing joints (Sridhar et al., 2012; Teichtahl et al., 2012; Lee et al., 2012).
1.3.2 Orthobiologics

A relatively recent and very exciting area of science and medicine is the advent of orthobiologics. By definition, orthobiology is the incorporation of biology and biochemistry in the development of bone and soft tissue replacement materials for skeletal and cartilage tissue healing. Regenerative medicine is important as it can stop or slow the progression of disease. With the advent of orthobiologics, orthopaedists have a number of options for increasing the strength and success of tissue repair while decreasing the length of the postoperative period. There has been a major impact in orthobiologics over the past few years thanks to improvements in biological and cellular therapies (Cole and D’Amato, 2001; Cancedda et al., 2003; Chen et al., 2006; Chung and Burdick, 2008; Fortier et al., 2010; Coccia, 2012; Danisovic et al., 2012). The field of orthopaedics involves treatment of diseases and injuries of the musculoskeletal system. These include mainly the treatment of sports injuries, back surgery, traumas and joint arthroplasty. Non-surgical treatments for OA involve preventative measures including physiotherapy and activity modification. Obesity is often a common factor for development of OA so weight loss can also be used to alleviate some of the stress on joints. Currently, non-pharmalogical and pharmalogical treatments such as steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are used for the early and moderately early treatment of OA. These measures however are only used to treat pain and inflammation. Protection or regeneration of healthy articular cartilage has not been demonstrated (Gerwin et al., 2005). Orthopaedists tended to use artificial metallic implants requiring very invasive operations and usually need replacement again later on in life (Long et al., 1998; Fortin et al., 2002; Baoqin, 2006). Biologic and cellular therapies help improve the efficiency of traditional tools and reduce the need for invasive surgeries (Coccia, 2012). This health sector is set to increase in importance as technology advances and as the average life expectancy increases.
The first widely accepted regenerative treatment for cartilage repair was autologous chondrocyte transplantation (ACT), more currently known as autologous chondrocyte implantation (ACI). Since its introduction in 1987, ACI has gained considerable attention for the management of full-thickness chondral defects of the knee and has renewed interest in cartilage repair. The ACI technique was initiated by Chesterman and Smith who first isolated and cultured chondrocytes free of the cartilage matrix (Chesterman et al., 1968.) Brittberg et al were the first to apply ACI in the clinic. The results of their first human clinical trial were published in 1994 (Brittberg et al., 1994). The widespread use of ACI began after Carticel® which is a preparation of autologous cultured chondrocytes (Genzyme Corporation, Cambridge, MA), became available in 1995 and the Food and Drug Administration (FDA) licensed it as a product in 1997. Briefly, ACI is a two-step process involving the harvest of a cartilage biopsy, processing and culture of autologous chondrocytes, and implantation of cultured cells (Brittberg et al., 1994; Cole and D’Amato, 2001; Minas et al., 1999; Peterson et al., 2002). There is also a variation called Matrix Associated Chondrocyte Implantation (MACI) which involves the use of a collagen biphasic scaffold to seed the chondrocytes onto (Behrens et al., 2005; Nuernberger et al., 2011). Chondrocytes are isolated from non-weight bearing zones of cartilage (Guerit et al., 2012). After cell cultivation, the autologous chondrocytes are loaded into the matrix. Typically re-implantation occurs three weeks after the harvesting procedure (Behrens et al., 2005; Nuernberger et al., 2011).

Currently there are patients with 10 to 13 years of follow-up who are benefitting from ACI (Minas et al., 2012; Park et al., 2012). The use of ACI requires special training, thus the cost of this service may vary according to the number of procedures performed, cell culturing, the cost included in shipping and the training of hospital staff. For example, Genzyme Ltd UK and Ireland charge £4000 to £5000 and BBraun/TeTec AG charge £4000; this however, may vary according to local agreements (National Institute for Health and Clinical Excellence, Technology Appraisal 89, 2008; www.nice.org.uk).
There are currently numerous clinical trials analysing the long-term benefits of ACI treatment (Bentley et al., 2012; Ebert et al., 2012; Mithoefer et al., 2012; Harris et al., 2011). However, a recent review carried out by Rodríguez-Merchán comparing the effects of ACI, mosaicplasty and microfracture concluded that there is no evidence of significant differences between these three treatments and based on the data reviewed it is possible that none of the treatment methods that aim to restore articular cartilage are actually effective (Rodríguez-Merchán, 2012).

Chondrocyte transplantation has other associated complications, such as donor site morbidity, repair cell de-differentiation (Benya et al., 1978; Diaz-Romero et al., 2005) with expansion in vitro, restricted cellular life span upon implantation (Brittberg et al., 2003; Guerit et al., 2012), spontaneous osteonecrosis after ACI treatment (Von Keudell et al., 2011) and there was increased treatment failures observed in subchondral bone defects that had prior treatment. These failed at a rate three times that of non-treated defects after ACI treatment (Minas et al., 2009). Another issue is the poor differentiation potential of OA-derived cells (Tallheden et al., 2005).

Other treatment options for OA include debridement, marrow stimulation techniques (abrasion chondroplasty, microfracture, drilling), osteochondral allograft and osteochondral autograft (Browne et al., 2000; Mandelbaum et al., 1998; Sgalione et al., 2002; Fujita et al., 2012; Donnenwerth and Roukis, 2012). Although the chondrocytes can be isolated from non-weight bearing zones of cartilage and expanded before implantation, the aforementioned issues and morbidity occurring at donor site leading over time to osteoarthritic lesions lead to the requirement for new cell sources for cartilage repair. MSCs are promising candidates (Wakitani et al., 1994; Bruder et al., 1998; Barry et al., 2001; Barry and Murphy, 2004; Mauck et al., 2006; Buckley et al., 2010; Hwang et al., 2011; Guerit et al., 2012). However, the conditions for effective isolation, induction of chondrogenesis and for production of a stable cartilaginous tissue after implantation are still to be optimized fully. Optimization of a stable tissue would be aided by the isolation of a more homogenous population of chondrogenic MSCs.
1.3.3 Immunogenicity of MSCs Provides Therapeutic Application in OA

MSCs exhibit immunosuppressive properties which make them a focus in the treatment of inflammatory disorders, such as rheumatoid arthritis (RA), which is the most common inflammatory autoimmune rheumatic disease. Inflammation in OA also leads to progression of tissue damage (Goldring and Goldring, 2007). The therapeutic use of MSCs has also been investigated in various other diseases such as treatment of graft versus host disease (GVHD) and prevention of transplant rejection (Le Blanc et al., 2008; Hematti et al., 2008; Franquesa et al., 2012).

Collagen-induced arthritis (CIA) is one experimental mouse model used for RA. MSCs were tested in the CIA model. In several studies, the injection of bone marrow (BM), or adipose tissue-derived MSCs improved the clinical score of the treated mice. These effects were due to a decrease of inflammation and TNF-α or IFN-γ serum levels along with an induction of a regulatory T cell phenotype (Augello et al., 2007; Gonzalez et al., 2009). More recently, IL-6-dependent secretion of prostaglandin E2 (PGE2) by MSCs was reported to inhibit experimental arthritis (Bouffi et al., 2010). The immunosuppressive effects observed have been shown to occur mainly through the secretion of soluble factors. It has been postulated that other possible mediators are indoleamine 2,3-dioxygenase (IDO) (Spaggiari et al., 2008), heme oxygenase (HO)-1 (Chabannes et al., 2007) as well as the secretion of human leukocyte antigen (HLA)-G (Selmani et al., 2008), transforming growth factor (TGF)-β (Bartholomew et al., 2001), interleukin (IL)-6 (Nauta et al., 2006) and PGE2 (Nemeth et al., 2009).

MSCs migrate to areas where they can act locally; Bouffi et al demonstrated, MSCs migrated to the joint where they acted inside the inflamed synovium to decrease the proliferation and function of immune cells by secretion of inhibitory soluble factors. They also acted systemically to suppress the host immune response through shifts in the Th1/Th2 cell balance, indicating that MSC-induced immune suppression is not mediated by just one or by a unique mechanism (Bouffi et al.,...
2010). Thus the immunogenic nature of MSCs may play an important role in destructive inflammatory responses. Currently there are 256 clinical trials underway using MSCs for disease treatment including GVHD, myocardial infarct (MI), stroke, multiple sclerosis, asthma and chronic obstructive pulmonary disease (COPD) (http://clinicaltrials.gov). There are however, some contradictory reports showing immunogenic responses (Schu et al., 2012) and in an earlier experimental model of RA, conflicting results in the first study on the use of MSCs in CIA showed allogeneic C3H10T1/2 cells did not actually exert a beneficial effect on disease progression (Djouad et al., 2005).

Sullivan et al described data comparing MSCs from different murine strains in CIA under identical experimental conditions that went some way to address the conflicting results. It was demonstrated that, despite their low immunogenicity, genetic mismatches between the MSC and host has a significant impact on the ability of MSCs to alter the progression of disease. Also the effect of MSCs on disease progression appeared to be mediated by alterations in host IL-17 and IL-1β production (Sullivan et al., 2012). This work suggested that further studies looking at the effect of varying cell numbers and repeated delivery of MSCs are required and there is information on MSCs and their immunogenicity which remains to be elucidated. The following figure (1.4) outlines the MSC’s immunomodulatory role.
Figure 1.4: Immunomodulatory role of MSCs; Schematic representing MSC activation by pro-inflammatory signals and the factors subsequently released by MSCs which induce pro-tolerogenic effects on components of the immune response (English et al., 2010).

1.4 Isolation of MSCs from Various Tissues for use in Orthobiologics

The microenvironment in which stem cells reside potentially influences their fate (Watt et al., 2000). Adult stem cells are found and have been isolated from a number of different tissues (Friedenstein et al., 1966; De Ugarte et al., 2000; Arai et al., 2002; English et al., 2007; Pei et al., 2008; Maxon et al., 2012). The isolation method used for MSCs results in cells that are poorly defined and give rise not only to a heterogeneous MSC population but also to osteoblasts, fat cells, reticular cells, macrophages, and endothelial cells (Seshi et al., 2000; Simmons and Torok-Storb,
As outlined in section 1.2.4 the isolation of a more homogenous population of cells would benefit in therapies using the application of MSCs for specific tissue repair.

The periosteum is one source of multipotent stem cells. Adult periosteal derived multipotent progenitors retain the ability to form bone, fat and cartilage (Nakahara et al., 1990; Nakahara et al., 1990; Nakahara et al., 1991; De Bari et al., 2001; Sakaguchi et al., 2005). Regardless of the age of the donor, periosteal progenitors are clonogenic and have significant in vitro expansion potential (De Bari et al., 2006) with continued positive expression of traditional BM-derived progenitor cell markers such as CD105, CD166, CD90, CD73 and CD44 (Arai et al., 2002). The presence of specifically chondro- and osteogenic precursors within the periosteum has been identified which makes this tissue an attractive tissue source for chondroprogenitor isolation (a more homogeneous chondrogenic population of cells) (Nakase et al., 1993).

The mature synovial membrane, a thin, weak layer of tissue lining the non-cartilaginous surfaces within an articulating joint, is composed of a fibrous external layer and an inner secretory layer that produces synovial fluid. Synovial tissue-derived multilineage progenitors may have the optimal therapeutic potential to regenerate damaged cartilage due to their capacity for proliferation and their superior chondrogenic differentiation potential (De Bari et al., 2001; Sakaguchi et al., 2005; Pacifici et al., 2006; Pei et al., 2008; Ando et al., 2008). Synovial membrane derived mesodermal progenitors, are not depleted in number or potential with donor age (De Bari et al., 2001). Interestingly, during the early stages of OA there is an increase in progenitor cell numbers in synovial fluid, presumably as a result of synovial membrane degradation leading to their release to the adjacent synovial fluid (Jones et al., 2008).

Infrapatellar fat pad (IFP) also contains a readily abundant source of CD105-positive, CD44-positive and CD166-positive progenitor cells with the potential to differentiate into mesodermal lineages (Jones et al., 2002). CD271 expression, a
putative marker of the *in vivo* progenitor cell (Jones et al., 2002), is highly expressed on fat pad derived progenitors and is uniquely maintained with proliferation, indicating retention of their progenitor capacity upon expansion (English et al., 2007). English et al., also demonstrated that 4±7% of the BM derived MSC population express CD271, whereas 31±17% of human adipose derived progenitors are CD271 positive, indicating the enrichment in progenitor cells in these heterogeneous cell preparations. The highly clonogenic progenitors present in IFP retained differentiative capacity and had a superior differentiative ability in OA derived IFP MSCs compared to BM derived MSCs (English et al., 2007). In fact, it has been demonstrated that IFP-derived cells are more similar to synovium-derived cells than to subcutaneous fat-derived cells (Mochizuki et al., 2006) and as outlined above, synovial tissue-derived multilineage progenitors may have one of the greatest overall therapeutic potential to regenerate damaged cartilage due to capacity for proliferation and superior chondrogenic differentiation potential (De Bari et al., 2001; Sakaguchi et al., 2005; Pei et al., 2008; Ando et al., 2008). Buckley et al demonstrated how porcine IFP-derived MSCs can undergo robust chondrogenesis and proliferation when encapsulated within agarose hydrogels (Buckley et al., 2010). Taken together, these studies describe IFP-derived MSCs as an autologous reparative cell source for the IFP-repair and regeneration of arthritic cartilage.

Adipose tissue-derived MSCs have been shown to differentiate into multiple lineages, such as cartilage, bone, fat, skeletal, smooth, and cardiac muscle, endothelium, hematopoietic cells, hepatocytes and neuronal cells. Subcutaneous fat can be harvested relatively easily with minimal morbidity or invasiveness and it contains many cells types such as adipocytes, preadipocytes, vascular smooth muscle cells and vascular endothelial cells as well as putative MSCs (De Ugarte., 2003). When comparing the chondrogenic ability of BM-derived MSCs and adipose-derived MSCs, Winter et al. demonstrated that the adipose-derived cells were in fact slightly compromised and had less complete chondrogenesis than the BM-derived cells (Winter et al., 2003). However; overall due to the frequency of adipose-derived MSCs, their relative ease of extraction and their ability to
differentiate make these cells a potential valuable resource for biotechnology and regenerative medicine.

Progenitor cells have also been identified in tendon, specifically in an ECM-rich niche. The tendon-derived stem/progenitor cells (TSPCs) are clonogenic with a higher rate of proliferation compared to BM-derived MSCs. TSPCs are multipotent with an enhanced potential for osteo- and adipogenic differentiation compared to BM-derived progenitors. With diminished chondrogenic potential and proficient tenogenic potential, TSPCs are perhaps best suited for tendon repair applications (Bi et al., 2007) promoting the idea that repair or regeneration is associated with tissue specific progression of stem cells.

Efforts by Friedenstein and colleagues (Friedenstein et al., 1966), Caplan (Caplan, 1991) and Pittenger and colleagues (Pittenger et al., 1999) unequivocally demonstrated the presence and potential of BM-derived MSCs with the innate ability to differentiate into multiple mesenchymal lineages in vitro. Despite their rare occurrence (0.001 to 0.01% of cells in the stromal compartment [Pittenger et al., 1999]), BM-derived MSCs may be efficiently isolated and expanded in culture without associated donor site morbidity. Adherent progenitors, CFU-Fs, initiate the generation of a clonal population of CD105-positive, CD73-positive, CD90-positive and CD44-positive cells that are then expanded in monolayer (Dominici et al., 2006). When comparing human BM and synovial-derived MSC, Djouad et al concluded that BM-derived and synovium-derived MSCs shared similar phenotypic and functional properties. Their capacities for chondrogenic differentiation were very similar (Djouad et al., 2005).

BM-derived MSCs are isolated non-selectively by exposing the mononuclear cell aspiration to tissue culture plastic. There is a requirement to optimize the isolation and ex vivo culture of MSCs in order to isolate as many functional cells as the marrow contains (Neuhuber et al., 2008). Adherence to tissue culture plastic is the oldest and most popular isolation method. It is common to allow the cells 5 days to adhere (Colter et al., 2000; Jiang et al., 2002; Dominici et al., 2006). The large-scale
isolation of MSCs from BM has not been completely realised. There is therefore a need to drive down the cost of marrow and stem cell processing by making the final product a more defined and more potent population while also maximising yields retrieved from the marrow. Other methods have been described to isolate MSCs from human BM such as the use of immunomagnetic beads and density gradient separation using Ficoll or Percoll centrifugation (Pittenger et al., 1999; Dennis et al., 1992; Jiang et al., 2001; Lennon et al., 2001; Stenderup et al., 2002). Several MSC protocols use extremely high numbers of MSCs (up to 5 million cells/kg body weight) for MSC transplantation in clinical use. The identification of “optimal” conditions for in vitro cell culture requires investigation. Recently studies have been analysing BM plating using varying plating densities (Mareschi et al., 2011) and using serum-free growth conditions (Jung et al., 2012).

Clonal analysis of BM-derived MSCs describes 20 to 50% of the total population of cells to be truly tri-potent, but most importantly has identified a sub-population of MSCs that retains only chondrogenic potential, or chondroprogenitors (Russell et al., 2010). As the resultant population of MSCs is heterogeneous, efforts continue to identify and isolate a homogenous chondroprogenitor cell population from marrow that would more efficaciously repair diseased cartilage.

### 1.5 Chondroprogenitors

Isolation of a more homogeneous chondrogenic population of cells (chondroprogenitors) may be necessary for more efficacious cartilage repair. Mesenchymal cell differentiation into chondrocytes and the associated deposition of ECM is minutely co-ordinated by paracrine factors. Through Sox9 as a transcriptional mediator, transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signalling are responsible for initiating expression of cartilaginous ECM such as aggregcan, collagen types II and XI, fibronectin and tenascin as shown in in vitro murine micro mass cultures (Hatakeyama et al., 2004; Chimal-Monroy and Diaz de Leon, 1999). Chondrogenic differentiation of adult
human progenitor cells in vitro through TGF-β1 is mediated by both Smad3 and Wnt-associated β-catenin (Church et al., 2002). In a clinical application, the ideal source of cells would be accessible, easily isolated and abundant, with a very minimal risk to the patient. So far with regards to clinical cartilage regeneration it appears that a more fibrocartilagenous type cartilage is formed which does not have the same structure, strength or durability as articular cartilage, making repair as of yet an elusive goal (de Vries-van Melle et al., 2012). Fibrocartilage is more of a temporary solution to damaged cartilage. It is a tough, fibrous material but it is not as strong or resilient and has less compressive ability compared to hyaline cartilage and fails to yield a long-term solution to tissue damage (Minas et al., 1997).

There are particular features that will be necessary to produce viable and durable cartilage tissue. These features include predominance of collagen type II, appropriate water content and bonding to the subcondral plate and surrounding cartilage matrix. Therefore the long term success of cartilage repair will depend on the restoration of articular cartilage that closely duplicates the normal cartilage (Newman, 1998). As the mature articular cartilage develops from embryonic mesodermal precursors that differentiate into chondroprogenitors (cells predisposed to differentiate into articular chondrocytes) and ultimately into mature adult chondrocytes or synoviocytes, it is hypothesized that progenitors retained in these adult articular tissues provide a potential reservoir of chondroprogenitors.

Immature progenitor cells with the potential to develop into specific, mature tissues in response to appropriate cues have become a primary focus of cartilage repair strategies as an alternative to chondrocyte-based methods such as the aforementioned ACI (Seo and Na, 2011). The application of chondroprogenitors, to repair articular lesions and subsequently inhibit the onset of OA is a current focus of research efforts.
1.5.1 Therapeutic Applications of Chondroprogenitors

Articular chondrocytes, when explanted and expanded *in vitro*, lose their chondrocytic phenotype as indicated by morphometric changes and elimination of collagen type II deposition (Wakitani et al., 2008). The re-differentiation of these cells *in vitro* regenerates their articular phenotype while the differentiation of a MSC *in vitro* results in the generation of a transient, pre-hypertrophic chondrocyte, similar to the chondrocyte phenotype in the developing embryonic skeleton (Winter et al., 2003), highlighting the inherent difference between progenitors and native chondrocytes (Huang et al., 2008; Hillel et al., 2010; Sabatino et al., 2012).

BM-derived MSCs have been directly injected (Murphy et al., 2003; Horie et al., 2012) or combined with a scaffold and implanted into the intra-articular space *in vivo* (Liu et al., 2006; Koga et al., 2008; Zheng et al., 2010) in an effort to assess their potential for efficacious repair of damaged cartilage tissue or diseased joints. These results have proven to be ambiguous and unsatisfactory as a result of low viability and retention of the cells (Murphy et al., 2003). More recently, Horie et al. demonstrated inhibition of OA however, this was in conjunction with rapid reduction in cell numbers (Horie et al., 2012). Due to inconsistency in results and association with hypertrophy and ossification, there are very few human clinical trials investigating heterogeneous bone marrow-derived MSCs as a therapeutic for cartilage repair.

The on-going Chondrogen clinical trial is currently investigating the application of BM-derived MSCs to treat meniscal damage and thereby delay the onset of OA. Conducted by Osiris Therapeutics, preliminary reports have claimed a statistically and clinically significant improvement in pain experienced by patients post-injury with application of MSCs [ClinicalTrials.gov: A Phase I/II Study of Chondrogen Delivered by Intra- Articular Injection Following Meniscectomy [http://www. clinicaltrials. gov/ ct2/show/ NCT00225095? term=chondrogen&rank=2]). The application of Chondrogen was well tolerated by recipients and superior to currently available, comparable products on the market. With advances in chondroprogenitor cell isolation and
culture techniques, products such as this will be improved upon by replacing large numbers of perhaps minimally efficacious heterogeneous MSCs with low numbers of highly efficacious chondroprogenitors.

To treat chondral defects, Advanced Technologies and Regenerative Medicine is currently investigating a cartilage autograft implantation system where autologous healthy cartilage is harvested from non-weight bearing regions, minced and re-distributed on a scaffold for implantation. Initial results have been promising, supporting a phase III clinical investigation (ClinicalTrials.gov: Cartilage Autograft Implantation System (CAIS) for the Repair of Knee Cartilage Through Cartilage Regeneration [http://www.clinicaltrials.gov/ct2/show/NCT00881023?term=CAIS&rank=1]). If left untreated, lesions such as these regularly result in the onset of OA. It is possible that the reparative cell responsible for the generation of neocartilage in this trial is indeed the re-implanted chondroprogenitor residing on the superficial surface of the harvested cartilage tissue. By identifying the reparative cell in this application, a less invasive methodology for reparative cell isolation could be developed, thereby greatly reducing donor site morbidity as well as enhancing the efficacy of the therapy. The current clinical interventions do not actually stimulate the generation of a mechanically sound reparative tissue, but focus on the short-term relief of OA symptoms, the field now looks to progenitor cell-based therapies as our future; specifically, progenitor cells primed for chondrogenic differentiation. The clinical application of a homologous chondroprogenitor population will eliminate the need for heterogeneous cell therapies (Ankrum et al., 2010) and result in an efficacious, minimally invasive approach to articular cartilage repair.

1.6 MSCs: Isolation and Expansion

A relatively small amount is known about the mechanisms, both cellular and molecular, that are underlying the control of MSC processes such as proliferation, survival and differentiation. As outlined by Ankrum, Karp and Chen et al, this lack of knowledge presents difficulties in characterising cells (Ankrum and Karp, 2010;
Chen et al., 2010). This is due to the inability to isolate and obtain a sufficient number of homogeneous MSCs using the typical culturing systems for \textit{in vitro} expansion. It is becoming increasingly evident how important the ECM is as a component of the cellular niche in tissues. It supplies biochemical and physical signals which are critical to initiate and sustain cellular functions.

\subsection{1.6.1 Cell-Extracellular Matrix Interactions}

The ECM has an important role in modulating the bioactivities of growth factors and cytokines; growth factors can be sequestered and inhibited from binding to their receptors or alternatively ECM can directly affect the growth factor receptors (Hildebrand et al., 1994; Santra et al., 2002). Not only do cells receive cues from the ECM but they also reciprocate by secreting ECM components and enzymes which cause proteolytic modifications of proteins and growth factors within the ECM. Structures of the ECM are subject to hierarchic organisations, these are tightly adapted to the functions of specific tissues and organs. Very few specialised tasks are reserved for isolated ECM macromolecules. Instead, molecular ECM components acquire their prominent functions only after polymerising into insoluble suprastructural elements such as fibrils and microfibrils, or networks that, in turn, are assembled into regional tissue structures, such as fibres or basement membranes. Most, if not all, ECM suprastructures are co-polymers of more than one molecular species that differ with respect to their identity and relative abundance. The understanding of these complex interactions particularly at the tissue level is not fully complete (Bruckner, 2010). These interactions represent a give and take relationship that defines the behaviour of cells (Behonick and Werb, 2003). The matrix has physical as well as biochemical functions; it is a connective macromolecular assembly which gives a tissue its shape and organises the cells within it. ECM comes in hugely varying forms as it is present in a large range of diverse tissues ranging from teeth, to tendons, to the cornea (Knudsen, 2003). The cells themselves produce the meshwork of macromolecules; it is thus likely associated with the cells by surface interaction and works as is required for the
specific tissues and the network in general (Dityatev and Seidenbecher et al., 2010). ECM is an important substrate for cell-cell communication and is suited to present signalling molecules for guiding cells.

Components of the ECM include fibrous protein such as collagen and elastin, proteoglycans attached to GAGs and glycoproteins such as fibronectin and tenascins (Faissner, 1993; Bandtlow and Zimmerman, 2000). GAGs consist of long, unbranched, repeating disaccharide units and are generally classified with respect to their disaccharide composition; it is the disaccharide formulation that distinguishes between chondroitin sulfate (CS), heparan sulfate (HS), keratin sulfate (KS), dermatan sulfate (DS) and hyaluronan (HA). None of the aforementioned GAG molecules are specific to one particular type of ECM. The disaccharide units themselves can be modified in various ways such as carboxylation or sulfation (Bulow and Hobert, 2006). The interaction between the highly negatively charged cartilage proteoglycans and type II collagen fibrils contributes to the compressive and tensile strength of cartilage. The ECM composition (Figure 1.5) that makes up the cartilage tissue are the collagen fibres, laminin polymers, cell adhesion proteins such as fibronectin, high molecular weight proteoglycans and growth factors of various types (often in the latent form) and members of small leucine-rich proteoglycan (SLRP) family - mainly biglycan and decorin (Clark and Keating, 1995; Hocking et al., 1998; Lee et al., 1999).

The unique composition of the disaccharides and the large number of posttranslational modifications make GAGs truly information dense biological molecules (Turnbull, Power et al., 2001). Due to their molecular structure, GAGs are suitable for binding many different signalling molecules. Hyaluronic acid (HA) is an exception because it is not protein bound, is non-sulfated, and is made of identical disaccharide units (Toole, 2001). HA is synthesised in the plasma membrane and forms the backbone of the cartilage ECM: most GAGs are covalently attached to a core protein. This is termed a proteoglycan.
Recently studies have revealed that the cells do not form direct contacts with the surrounding matrix but are surrounded by a thin sheet of pericellular matrix containing proteoglycans and thin collagenous filaments (Hunziker et al. 1998). Wu et al. (2009) have also reported that the collagenous composition of cartilage fibrils is altered with age and exact tissue localisation.

**Figure 1.5:** Cartilage extracellular matrix; A schematic outlining the components of the ECM including the singular chondrocytes surrounded by collagens (primarily type II collagen), proteoglycans (mainly aggrecan but also biglycan, decorin and fibromodulin), and other non-collagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein). COMP: cartilage oligomeric matrix protein (Chen et al., 2006).
It has been well established that BM ECM components play a critical role in differentiation of hematopoietic stem cells (Drzeniek et al., 1997). Less is known about the influences of the ECM on MSC differentiation however, some studies have led to strong indications that BM ECM also has an important role in MSC differentiation (Xu et al., 1998; Corsi et al., 2002; Hwang et al., 2011).

1.7 Specific Components of the ECM

1.7.1 Chondroitin Sulfate

CS is a major component of ECM and is important in maintaining the structural integrity of the tissue (Wu et al., 2010). This structural integrity function of CS is a typical feature of the large aggregating proteoglycans such as aggrecan, versican, brevican and neurocan. These are collectively known as lecticans. CS is composed of repeating disaccharide units of N-acetylgalactosamine (GalNAc) with glucuronic acid. It is a sulfated molecule and can be sulfated at different GalNAc residues (Bian et al., 2009; Chen et al., 2011). Sulfation patterns on CS are associated with its specific properties. CS is tightly packed and highly sulfated and thus highly charged. This generates electrostatic repulsion that provides much of the resistance of cartilage to compression (Bian et al., 2009).

Chondroitin-6-Sulfate (C6S) is composed of glucuronic acid with 90% of the GalNAc residues sulfated at the carbon 6 position. C4S is sulfated at the carbon 4 position. It is the presence of these specific sulfated motifs within the GAG chains that allow binding and regulation of signalling molecules (Figure 1.6). This in turn regulates intracellular signalling pathways which drive cell behaviours such as proliferation, differentiation and matrix synthesis (Tiedemann et al., 2005).

CS has useful biological properties for use in cartilage engineering. These properties include anti-inflammatory activity, water and nutrient absorption, wound healing
and activity at the cellular level that can help restore arthritic joint function (Pipiton et al., 1991; Roneo et al., 1998; Li et al., 2004).

Figure 1.6: (A) Molecular structure of CS sulfated at the carbon 4 position, Chondroitin-4-Sulfate and (B) sulfated at the carbon 6 position, Chondroitin-6-Sulfate (Sobal et al., 2008).

Chondrocytes cultured on CS modified chitosan membranes were shown to retain their phenotype and to produce cartilage specific matrix in a previous study (Sechriest et al., 2000). Huang et al, has also reported a modulatory effect of extracellular CS on chondrocytes (Huang et al., 1977) and a stimulatory effect of CS on proteoglycan and GAG production in cartilage ECM has also been shown (Huang et al., 1974; Huskisson, 2008). Importantly, CS is highly expressed during the pre-cartilage condensation of MSCs, suggesting its importance in chondrogenesis (Kamiya et al., 2006; Barry et al., 2001).

CS is commonly given to OA patients as a dietary supplement, along with glucosamine. It can be used alongside pharmaceutical medications and painkillers
as an alternative treatment. It is approved and regulated as a symptomatic slow acting drug for OA (SYSADOA) as it may improve function, and reduce joint swelling and effusion (Jordan et al., 2003; Jomphe et al., 2008). A review by Martel-Pelletier et al. summarized the data relating to the mechanisms of action of CS in the pathophysiology of osteoarthritic joint tissues. This review suggested that the effect of CS on OA patients is possibly the result of stimulating synthesis of proteoglycans and decreasing the catabolic activity of chondrocytes by inhibiting the synthesis of proteolytic enzymes and other factors that contribute to matrix damage and chondrocyte death (Martel-Pelletier et al., 2010). The overall conclusion of the effect of CS on OA cartilage pathophysiology was that it is possibly due to its contribution to a balance between anabolism/catabolism in the articular tissues. In vitro studies have shown that the anti-inflammatory effect of CS is due to a reduction in IL-1β-induced nuclear factor-κB (NF-κB) translocation in chondrocytes (Jomphe et al., 2008). A recent clinical trial of orally administered CS to determine effects on cartilage volume loss, subchondral bone marrow lesions (BML), synovitis and disease symptoms in patients with knee OA demonstrated that CS treatment significantly reduced the cartilage volume loss in knee OA starting at 6 months of treatment, and BML at 12 months after initiation of treatment (Wildi et al., 2011).

In vitro studies have also been carried out using CS; the effect of a three-dimensional synthetic–biological composite hydrogel scaffold comprised of poly (ethylene glycol) (PEG) and CS on MSC chondrogenesis demonstrated that the synergistic action of the CS moieties of the scaffold along with TGF-β provided a micro-environment that is conducive for MSC chondrogenesis. Particularly, CS-based hydrogels facilitated the condensation of encapsulated MSCs followed by earlier expression of cartilage specific markers and subsequent matrix component production. They demonstrated that a synergistic action of CS on a PLGA scaffold with TGF-β can provide a micro-environment that is conductive for MSC chondrogenesis (Varghese et al., 2008). Another study carried out by Chen et al demonstrated that CS increased the chondrogenesis of MSCs while down-regulating osteogenic genes and cell proliferation which was consistent with
several other CS based studies on MSC chondrogenesis (Chen et al., 2011; Park et al., 2010; Uygun et al., 2009). These positive effects of CS on MSCs make it an interesting molecule to test its effects on the isolation of MSCs directly from the bone marrow.

### 1.7.2 Hyaluronan

HA is a non-proteoglycan polysaccharide and an unbranched, linear polymer of the repeating disaccharide 2-deoxy, 2-acetamido-D-glucopyranosyl-h (1, 4)-D-glucuronopyranosyl (Figure 1.7). HA is not a sulfated molecule nor is it covalently attached to protein and it is typically several hundred-fold larger than other glycosaminoglycan chains. The molecular weights of HA from different sources are highly variable, ranging from $10^4$ to $10^7$ Da (Liu et al., 2011). For normal differentiation processes, the ability of HA to form large aggregates in the ECM is necessary, it does this by binding to the resident proteoglycans. HA is present in the ECM in tissues of every vertebrate from the vitreous of the eye to the ECM of cartilage tissues (Fraser et al., 1997). It is a highly hydrated poly-anionic macromolecule and it is an essential component of the ECM, where its structural and biological properties mediate cellular signalling, morphogenesis, wound repair and matrix organization (Toole, 2001; Toole, 2004). HA has a rapid turnover in the body mediated by hyaluronidase, with a tissue half-life ranging from hours to days (Laurent and Fraser, 1986). Interestingly, the presence of this polysaccharide in the extracellular space confers upon tissues the ability to resist compression. It does this by absorbing significant amounts of water (Fraser et al., 1997).

HA has been given a lot of attention in the last few decades due to the fact that it has many physiological functions. Initially, it was assumed that HAs function was based on physical properties such as joint lubrication, tissue homeostasis and tissue adhesiveness. It is however, also a major regulator of cellular behaviour during processes such as embryogenesis, regeneration, morphogenesis, migration, proliferation, drug resistance and differentiation (Solis et al., 2012 and Chen et al.,
HA has also been clinically used as a medical product (Prestwich and Kuo, 2008) and has become an important building block for the creation of new biomaterials with utility in tissue engineering and regenerative medicine (Allison et al., 2006; Burdick and Prestwich, 2011; Prestwich, 2011).

Figure 1.7: Chemical structure of a disaccharide unit of hyaluronan. Typically HA is several thousands of sugars in length (Kapoor et al., 2011).

HA’s biological production has been linked to a variety of disease, developmental, and physiological processes (Toole et al., 1989; Toole et al., 1992; Laurent and Fraser, 1992; Knudson and Knudson, 1993; Laurent et al., 1995). The many functions depend on interactions with various cell surface receptors. These receptors include CD44, receptor for HA mediated motility (RHAMM), lymphatic vessel endothelial hyaluronan receptor (LYVE-1), hyaluronan receptor for endocytosis (HARE), liver endothelial cell clearance receptor (LEC-Receptor) and toll-like receptor 4 (TLR4). Hyaluronan-induced signalling occurs through receptor
interactions, however, HA signal transduction mechanisms are not fully characterized but have been shown to be a key influence on stem cell behaviour. This became apparent after its identification in many locations in which stem cells reside (Chen et al., 2007 and Liu et al., 2008). HA has been shown to aid the migration and proliferation of MSCs and chondrocytes via its association with specific cell surface receptors such as CD44 and RHAMM (Turley et al., 1993, Zhu et al., 2006, Toole, 2001).

Chen et al described that murine adipose-derived stromal cells (mADSCs) have a finite proliferative capacity and rapidly acquire a senescent morphology. Additionally, these mADSCs are highly sensitive to environmental stresses such as hyperoxic in vitro conditions and frequent sub-cultivation (Chen et al., 2007). These culture issues were also described in human MSCs by Matsubara et al. This study described that basement membrane ECM provided a more successful culture technique for MCSs (Matsumara et al., 2004). The Chen et al study also suggested that HA in the medium increased the growth of MSCs at early passages, extended their lifespan and reduced senescence during sub-passaging (Chen et al., 2007). These studies were carried out on passaged MSCs.

ECM provides a micro-environment for cells to maintain homeostasis and differentiation properties (Hunziker, 2002). HA is a major physiological component of articular cartilage matrix environment and is especially abundant in synovial fluid (Yoo et al., 2005). The knowledge that the cell’s micro-environment plays a critical role in controlling and guiding stem cell differentiation was used by Wu et al. to hypothesise that immobilizing HA on the surface of a biomaterial may provide an appropriate micro-environment for human ADSCs (hADSCs) to differentiate into the chondrogenic lineage and produce a cartilage-specific matrix for articular cartilage regeneration. These authors cultured hADSCs on HA-coated poly-lactic-co-glycolic acid (PLGA) bio-scaffold. Gene expression and ECM formation provided evidence that the HA/PLGA scaffold led hADSCs towards chondrogenesis (Wu et al., 2010). The ability of HA to create a chondro-inductive environment which promotes synthesis of cartilage tissue was also applied to other studies both in vitro and in vivo.
vivo where hyaluronan-based scaffolds have been found useful for inducing hyaline cartilage regeneration (Correia et al., 2011, Nehrer et al., 2006, Welsch et al., 2010).

The aggregation of chondroprogenitor MSCs into pre-cartilage condensations is an early event during limb development (Thorogood and Hinchcliffe, 1975). The presence of HA is critical for cell-to-cell cross bridging for cell aggregation prior to pre-cartilaginous condensations (Knudson, 2003). Hyaluronan also regulates signal transduction during the embryonic development of mesenchymal cells; this event leads to the formation of bone and cartilage (Astachov et al., 2011).

HA may induce faster cell attachment and enhance cell differentiation, through improved cell-cell communication (Jha et al., 2011). It remains unknown how HA mediates these processes, however, the HA mediated event has been confirmed by a new class of engineered HA-based hydrogels that provide a natural ECM environment with a complex mechanical and biomedical interplay. Without the use of osteogenic media, HA-bound hydrogels induced osteoblast differentiation of MSCs through enhanced cell adhesion (Jha et al., 2011).

HA hydrogels are useful for molecule delivery applications due to their excellent biocompatibility, non-toxic nature, and tenability of properties and degradation (Weiland et al., 2007). They have been used to control the differentiation of encapsulated stem cells, such as in cross-linked thiolated HA gels; thiol-modified macro-monomers spontaneously crosslink in air to form a hydrogel which can be dried to give a thin film or lyophilized to produce a porous sponge (Sherban and Prestwich, 2008). Similarly, photo-cross-linked HA hydrogels are proving to be promising for use in 3D stem cell encapsulation. In Chung’s study, MSC chondrogenic differentiation was investigated in photo-polymerized HA hydrogels. Both in vitro and in vivo cultures permitted chondrogenic differentiation, measured by increased early gene expression of collagen type II, aggrecan and SOX9 (Chung and Berdick, 2009). Upon assessing the importance of hydrogel chemistry on MSC chondrogenesis, the HA hydrogels were compared to inert PEG hydrogels in the
presence of chondrogenic factors such as TGF-β3. MSCs in HA hydrogels showed significantly enhanced expression of cartilage specific markers compared to the PEG hydrogels both in vitro and in vivo. Chung and Burdick’s work indicated that HA hydrogel chemistry can play a role in MSC differentiation and can enhance chondrogenesis.

HA has been injected into OA joints as a pain relief therapy. The goal of intrarticular injection is not only to relieve pain and to replace fluid which is lacking in viscoelastic properties, but also to possibly modify disease activity by HA’s molecular properties such as stimulation of chondrocyte growth, decreasing apoptosis and stimulating the production of cartilage matrix components such as endogenous HA, collagen and proteoglycans (Goldberg and Buckwalter., 2005).

HA can reduce pain via several mechanisms. It has been shown to bind to neuropeptides and creates a barrier around the nocireceptors which reduces pain. Another potential mechanism is the inhibition of inflammatory mediators such as cytokines and prostaglandin (Goldberg and Goldberg, 2010). HA has a very low residence time in the knee and it is cleared very quickly from the site of injection after exogenous administration (Mooreland, 2003). It was therefore unanticipated and fortuitous that the HA has such a positive therapeutic effect on the knee.

Another method by which HA helps to reduce pain is enhancing synovial fluid flow by restoration of metabolic homeostasis (Goldberg and Goldberg, 2010). Exogenous HA has also been shown to stimulate synthesis of endogenous HA by synovial sites through CD44 receptor binding (Kawasaki et al., 1999). All of these HA effects can restore homeostasis and reduce pain and stiffness.

Taken together, these highly important effects of both endogenous and exogenous HA on MSCs and physiological functions suggest that HA may be effective for isolation of chondroprogenitors from bone marrow.
1.8 ECM and the Stem Cell Niche

The term “niche” was first described in 1978 (Schofield et al., 1978). Studies in cell biology emerged in relation to the discovery of the niche, which included a focus on the micro-environment that supports stem cells. The stem cell niche involves the surrounding cellular components of the micro-environment and the associated signals emanating from these support cells; it is not just the location where these cells are present (Li et al., 2005). The niche requires a balanced environment that controls the fate of the cells between self-renewal and differentiation. Absence of the balancing environment triggers inappropriate differentiation (Solis et al., 2012). Niches are specific anatomic locations which regulate how stem cells participate in repair, regeneration and maintenance (Scadden et al., 2006).

Recent studies that analysed the stem cell niche have revealed that cell types such as endothelial cells, osteoclasts and mesenchymal progenitors are imperative in establishing function. The pressure on the stem cell’s metabolic activity compared to other cells in the body may necessitate special support and sustenance from their micro-environment. There is also the possibility that the cells require feedback control because stem cell pools are usually capable of expanding and contracting and can sometimes face large stochastic fluctuations under certain homeostatic conditions (Vazin and Schaffer, 2010; Morrison et al., 2008; Lam et al., 2006).

The use of ECM molecules during the isolation of MSCs from bone marrow may provide a bio-mimetic environment for the MSCs to receive cues as they would in the developing joints in vivo. This in turn may lead to the production of a more stable, hyaline cartilage for use in regeneration of degenerated tissue.
1.9 Thesis Objectives

OA or degenerative joint disease is the most prevalent of all musculoskeletal diseases and a leading cause of morbidity. To date, there are no effective treatment options to repair or regenerate damaged articular cartilage and ultimately, OA leads to a total joint replacement. Thus, there is a need to develop methods that are less invasive and capable of regenerating articular cartilage. The use of autologous chondrocytes in ACI has a number of limitations in terms of efficacy and safety as discussed earlier. As a result, MSCs are considered an alternative therapeutic candidate for chondral repair. The ease of MSC isolation from a number of tissue sources, their high proliferative capability and ability to differentiate into chondrocytes in vitro make them appealing substitutes for chondrocytes in cartilage regeneration.

However, in vivo MSCs are a rare occurrence within the bone marrow (0.001 to 0.01% of cells in the stromal compartment [Pittenger et al., 1999]). BM-derived MSCs are generally isolated non-selectively by exposing the mononuclear cell aspiration to tissue culture plastic. There is a need to optimize the isolation and ex vivo culture of MSCs in order to isolate as many functional cells as the marrow contains (Neuhuber et al., 2008). Clonal analysis of BM-derived MSCs has identified a sub-population of MSCs that retain only chondrogenic potential, otherwise known as chondroprogenitors (Russell et al., 2010). As the general methods of MSC isolation produce a population of heterogeneous MSCs, the aim of this thesis is to identify and isolate a homogenous chondroprogenitor cell population from marrow that could be used to more efficaciously repair diseased cartilage. Therefore the overall objective of this thesis was to isolate a chondroprogenitor cell population directly from bone marrow using cartilaginous ECM in order to create a more efficient method of obtaining cells for the treatment of OA.

To achieve this objective, the thesis was divided into the following aims:
Aim 1.

Identify the most efficient cartilage ECM molecules to isolate a chondroprogenitor cell population from human bone marrow.

Previous studies have shown that the use of ECM molecules with MSCs has enhanced cell proliferation, and production and deposition of matrix components (Cristino et al., 2005; Pasquinelli et al., 2008). MSC incorporation onto ECM-based scaffolds is also known to induce chondrogenic differentiation and deposition of a cartilage-like ECM (Jakobsen et al., 2010; Chung et al., 2009).

The hypothesis was that there is a sub-population of progenitor cells in BM that are primed towards the chondrogenic pathway with pre-requisite receptors for cartilage ECM molecules. Furthermore, these chondroprogenitors can be isolated from bone marrow via their specific adhesion to these cartilaginous ECM molecules. This was carried out by adsorbing the cartilaginous ECM molecules to tissue culture plastic before direct plating of the BM for 1 and 5 days. Results for these experiments are summarized in chapter 2

Aim 2.

Analyse the non-adherent bone marrow populations for MSCs after 1 and 5 days exposure to cartilage ECM molecules and identify the most efficient time-point for exposure and ECM selector for chondroprogenitors.

The objectives are to assess the non-adhered populations for putative MSCs, to compare these cells to those isolated by traditional methods and the adherent ECM-isolated populations described in chapter 2, and to compare these non-adherent sub-populations (both the early sub-population and late sub-population) to all other aforementioned MSC populations for differentiation potential. More specifically, non-adherent sub-populations will be assessed for depletion of chondroprogenitors.
These objectives will be achieved by analysis of the non-adherent bone marrow populations (those remaining non-adherent after the 1 day and 5 day time-point) for viable MSCs.

Aim 3.
Assess whether direct exposure of in vivo chondroprogenitors in bone marrow to ECM molecules will enable isolation of the cells

The hypothesis was that there are methods of exposure other than ECM coating onto the flasks that promote chondrogenesis of marrow chondroprogenitors. The objective was to analyse if it would suffice to add the ECM molecules directly to the whole bone marrow before BM processing and MSC isolation.

Aim 4.
Identify the potential presence of a synergistic relationship existing between the BM and ECM molecules that is required for the ECM to have its selective effect on chondroprogenitors

If results from previous chapters demonstrate an increase in chondrogenesis with the use of ECM molecules on MSC isolation as compared to ECM exposure to passaged cells, it may be hypothesised that the molecular mechanisms underlying the activation of MSCs can be mediated by signals from the marrow environment, and that the specific cytokines or paracrine factors in the ECM micro-environment interact with the MSC surface and activates an MSC fate decision.

The hypothesis thus proposed is that there is a synergistic effect between the ECM molecules and the marrow niche that enhances the effect of ECM molecules on MSCs in the marrow, thus contributing to the enhancement in chondrogenic potential.
Chapter 2

Identification of Cartilage ECM Molecules for the Isolation of a Chondroprogenitor Cell Population from Human Bone Marrow.
2.1 Introduction

Prevalence of OA is expected to rise dramatically over the coming 20 years with the consistent increases in life expectancy within the general population. This creates an imperative for the timely development of effective treatments for the disease (Bergman et al., 2007). Current clinical therapies such as pharmaceutical interventions, bone marrow stimulation techniques or micro fracture do not result in the regeneration of healthy cartilage tissue (Qvist et al., 2008; Mithoefer et al., 2009), but focus on the short-term relief of OA symptoms or generation of fibrous tissue (fibrocartilage) which does not have the same tensile strength or longevity as healthy hyaline cartilage (Mithoefer et al., 2009). When intervention fails, clinicians regularly revert to invasive and permanent solutions such as total joint replacement.

The first widely accepted regenerative treatment for cartilage repair was autologous chondrocyte transplantation (Brittberg et al., 1999). Despite its initial therapeutic promise, chondrocyte transplantation has shown associated complications such as donor site morbidity, repair cell de-differentiation with expansion in vitro and restricted cellular life span upon implantation (Brittberg et al, 2003). Immature progenitor cells, or chondroprogenitors, have the potential to develop into mature tissues in response to appropriate cues and have therefore become a primary focus of cartilage repair strategies as an alternative to chondrocyte-based methods (Alberts et al., 4th Edition). Chondroprogenitors are cells that are specifically pre-disposed to differentiate into mature chondrocytes and to repair articular lesions in OA and other degenerative joint diseases. The mature articular joint develops from embryonic mesodermal precursors that differentiate into chondroprogenitors and ultimately into mature adult chondrocytes or synoviocytes (Pacifici et al., 2006). It is hypothesized that progenitors retained in these adult articular tissues provide a potential reservoir of chondroprogenitors. However, the numbers of endogenous chondroprogenitors
available in adult cartilage is below the level necessary to treat degeneration and combat disease. This has led to the need to identify progenitor sources elsewhere in the body that have a stem cell reserve with ease of access, expansion capacity and chondrogenic potential. Over the past decade, the BM has been studied as a rich progenitor source as it possesses a MSC population with the prerequisite characteristics set out above (Barry et al., 2001; Barry, 2003; Barry and Murphy, 2004; Augello et al., 2007; Guerit et al., 2012). One of the key aims of this thesis is to develop a process which enables a putative sub-set of chondroprogenitors to be isolated from BM in vitro. The enrichment of this subset from BM directly, will allow novel chondroprogenitor based therapeutics to be developed for OA cartilage repair.

Cartilage is a tissue composed primarily of ECM surrounding chondrocytes. ECM regulates the behaviour of the cells that move near it, inhabit it or move through its meshes (Alberts et al., 4th Edition). The cartilage ECM is composed of numerous macromolecules, namely, proteoglycans (such as perlecans, aggrecan, versican, brevican and neurocan), collagens, elastin and non-collagenous glycoproteins. As part of the aggrecan proteoglycan, CS is a major component of ECM and is important in maintaining the structural integrity of the tissue (Wu et al., 2010). It is tightly packed and highly sulfated and thus highly charged. This generates electrostatic repulsion that provides much of the resistance of cartilage to compression (Bian et al., 2009). It is the presence of these specific sulfated motifs within the GAG chains that allow binding and regulation of signalling molecules which in turn regulates intracellular signalling pathways which drive important cell behaviours (Tiedemann et al., 2005). HA is a non-proteoglycan polysaccharide. For normal differentiation processes, the ability of HA to form large aggregates in the ECM is necessary, it does this by binding to the resident proteoglycans. HA is present in the extracellular matrix in tissues of every vertebrate, it confers upon tissues the ability to resist compression. It is an essential component of the ECM, in which it’s structural and biological properties mediate its activity in cellular signalling, morphogenesis, wound repair and matrix organization (Toole, 2001; Toole, 2004).
It was therefore hypothesised that there is a sub-population of progenitor cells in BM that are primed towards the chondrogenic pathway with pre-requisite receptors for cartilage ECM molecules. Furthermore, these chondroprogenitors can be isolated from bone marrow via their specific adhesion to cartilaginous ECM molecules.
2.2 Materials and Methods

All materials were supplied by Sigma Aldrich unless otherwise stated.

2.2.1 Isolation of Human MSCs

hMSCs were isolated from the BM of the iliac crest of healthy donors; all procedures were performed with informed consent and ethically approved by the Clinical Research Ethical Committee at University College Hospital, Galway. hMSCs were isolated and expanded in culture by direct plating, as previously described (Murphy et al., 2002). Briefly, approximately 25 ml of BM aspirate was obtained in heparin from each donor. The aspirate (5 ml) was aliquoted into 50 ml sterile centrifuge tubes and 45 ml Dulbecco’s phospho-buffered saline (D-PBS; Gibco) added. The BM suspension was centrifuged at 900 x g for 10 minutes and the supernatant was gently aspirated off and discarded taking care to leave the pellets undisturbed. Five ml of D-PBS in total was used to sequentially re-suspend the mononuclear cell pellets before transfer to a fresh 50 ml tube. A 50 μl aliquot of this suspension was taken and added to 450 μl of D-PBS. Of this suspension, 50 μl was added to 50 μl of 4% (v/v) acetic acid in a microcentrifuge tube to lyse the red blood cells. Total mononuclear cell number was determined using a haemocytometer and primary cells were plated at a cell density between 40 x 10^6 – 60 x 10^6 cells / T-175 flask (~4000-5000 MSCs/cm²). Complete hMSC medium [alpha-Minimum essential medium (α-MEM; Gibco) supplemented with 10% heat inactivated fetal bovine serum (HI FBS), 1% antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate), 1% non-essential amino acids (NEAA) and 5 ng/ml basic fibroblast growth factor (bFGF-2; R&D Systems) was added to the flasks to a final volume of 35 ml/T-175 flask. Cells were incubated under normal growth conditions; 37°C, 5% CO₂ and 90% humidity. On day 3, the BM cultures were topped up with a further 15 ml of complete hMSC medium. On day five the flasks were swirled to dislodge red blood cells and the medium was removed. After washing with 10 ml D-PBS, 35 ml of complete hMSC medium was added to each flask. Cells
were fed twice weekly and sub-cultured when colonies had formed and proliferated (approximately day 12-14). Subsequently, cultures were passaged at 5-7 day intervals and expanded to passage 3 (P3) for experimentation.

All cells used in this thesis are human BM-derived MSCs and will be referred to as MSCs in this thesis.

2.2.2 Sub-culture of hMSCs

Once the MSCs in culture had reached 80% confluence they were passaged. The medium was removed and the cells were washed twice with 5 ml D-PBS. The cells were detached from the tissue culture flasks by enzymatic action; 0.25% Trypsin/EDTA (5 ml per T-175 flask or 0.02ml/cm²) was added and flasks incubated at 37°C for 5 minutes, followed by sharp tapping to ensure the cells had detached. In order to quench the enzymatic action of the trypsin/EDTA, an equal volume of complete hMSC medium was added to the cells and the suspension was transferred to a sterile centrifuge tube. The cell suspension was centrifuged at 400 x g for 5 minutes at 25°C. The supernatant was aspirated and the cells were resuspended in 10 ml of complete hMSC medium. Total cell number was counted (Section 2.2.1) and re-plated at 6 x 10³ hMSC/cm².

2.2.3 Tissue Culture Plate Coating with ECM Molecules

Twenty four hours before the hMSCs isolation from BM, T-25 tissue culture flasks were coated with 5 ml of either 1 mg/ml sterile hyaluronic acid (HA) (Durolane [Smith & Nephew]) mixed in serum free medium (α-MEM without any supplementation) or 1mg/ml sterile chondroitin-6-sulfate (CS) or heparan sulfate (HS) dissolved in α-MEM. The flasks were then sealed with parafilm to preserve sterility and placed on a plate rocker at 4°C for 24 hours to ensure overall coating of the culture flasks. The following day, just before plating of washed BM
mononuclear cells, the coated flasks were briefly washed with 5 ml α-MEM to remove unbound ECM molecules (Figure 2.1).

**Figure 2.1: Extracellular Matrix Coating with ECM:** Schematic of the process of coating tissue culture plastic with CS and HA resuspended in serum-free medium.

### 2.2.4 Isolation of Early Adherent and Late Adherent MSC Populations

In order to obtain the early adherent MSC populations, the bone marrow and non-adhered cells were removed from the culture dishes after 24 hours. The adhered cells were washed twice with 5 ml of serum free medium (α-MEM without any supplementation) and received 7 ml fresh maintenance medium and left to grow to confluence. This population of cells was referred to as the early adherent population or EA population. The remaining flasks retained the bone marrow for the usual 5 day period and were then washed to remove non-adherent cells and
grown to confluence. This population was referred to as the late adherent or LA population (Figure 2.2).

Figure 2.2: Experimental Design; isolation of early and late adherent cell populations: The BM was removed after 24 hours from EA flasks to isolate early adherent cells. The BM was left on the remaining flasks for a further four days allowing for the isolation of late adherent (LA) cells. All flasks were expanded to 80% confluence and passaged onto non-coated tissue culture flasks for two passages at the end of P0. At the end of passage 2 the EA and LA isolated cells were induced towards chondrogenic differentiation in pellet format for 21 days, along with adipogenic and osteogenic differentiation.
2.2.5 Characterisation of Human MSCs

2.2.5.1 Adipogenesis

hMSCs were seeded into 4 wells of a 6-well plate at 200,000 cells/well (2 x 10^4/cm^2) in triplicate and expanded until cultures were ~90% confluent. Treated cultures were then placed in adipogenic induction medium (DMEM high glucose; 10% FBS; 1 μM dexamethasone; 10 μg/ml insulin; 200 μM indomethacin; 500 μM isobutylmethylxanthine; 100 U/mL penicillin and 100 μg/mL streptomycin) for 3 days while control wells received normal hMSC growth medium throughout culture. After 3 days of induction, the medium was changed on treated cultures to adipogenic maintenance medium (DMEM high glucose; 10% FBS; 10 μg/ml insulin; 1% penicillin/streptomycin) for a minimum of 1 day followed by 2 more induction/maintenance cycles.

Oil Red O staining of lipid: Oil Red O stock solution (0.3% of Oil Red O powder in 99% isopropanol) was diluted to a working solution by mixing 6 parts of Oil Red O stock solution with 4 parts of distilled water. After 10 minutes the solution was filtered using Whatman no.1 filter paper (Whatman). The maintenance medium was removed from the cultures and the well was washed twice with D-PBS and then fixed with 10% neutral buffered formalin for 20 minutes at room temperature. Following a wash with distilled water, the cells were covered with Oil Red O stain for 5 minutes. Upon removal of the stain, the wells were rinsed in 60% isopropanol followed by tap water. Cells were counterstained with haematoxylin for 1 minute and rinsed with warm tap water. Oil red O stained lipid droplets were visualised and photographed with a confocal microscope (Olympus IX71) with imaging software (Olympus cell^P). After imaging, the water was removed from each well. Oil Red O was extracted from lipid filled vesicles using 100% isopropanol and evaluated in a 96-well plate. One hundred per cent isopropanol was used as a blank. The extracted stain was measured using a Wallac Victor™ 1420 Multilabel Counter spectrophotometer capable of reading absorbance at 490nm.
2.2.5.2 Osteogenesis

MSCs were plated at 30,000 cells per well (2 x 10^4 cells/cm^2) of a 6-well plate and were allowed to adhere for 24 hours. Control wells were fed throughout culture with complete hMSC growth medium while the other 3 wells were treated with osteogenic medium (DMEM low glucose; 10% FBS; 100 nM dexamethasone; 50 μM ascorbic acid 2-phosphate; 10 mM β-glycerophosphate and 1% penicillin/streptomycin). hMSCs were fed twice weekly and the plates assayed for calcium deposition at ~day 17.

For the quantification of mineral deposition, the Stanbio Calcium Liquicolour Kit was used. Osteogenically differentiated samples were briefly washed twice with D-PBS and 0.5 M HCl was then added to each well. The wells were scraped and the contents were placed into separate Eppendorf tubes. This was repeated once to ensure all matrix was removed from the wells. The samples were shaken overnight at 4°C and centrifuged at 3,000 rpm for 5 minutes. Standards were prepared according to manufacturer’s instructions with 0.5 M HCl and deionised water ranging from 0.05 μg to 1.5 μg. Stanbio Calcium (CPC) Liquicolor working solution (1:1 working dye to binding reagent) was added to standards and samples in a 96-well plate as per manufacturer’s instructions (200 μl per well) and incubated at room temperature, in the dark, for 15 minutes. Samples were assayed in triplicate and the absorbance was read on a Wallac Victor™ 1420 Multilabel Counter spectrophotometer at 550 nm.

Staining for calcium deposition in the osteogenic cultures was carried out using Von Kossa stain: the cultures were washed twice with D-PBS and fixed with 10% formalin for 20 minutes. Silver nitrate solution (3%) was added to each well and incubated at room temperature in the dark for 10 minutes. The wells were then rinsed three times in water where the last wash was exposed to strong, warm light for 15 minutes. These cultures were again washed twice with water and the stained matrix deposits were visualised and photographed using a confocal microscope.
(Olympus IX71) with imaging software (Olympus cell^P). A few ml of water was left in the wells to ensure they did not dry out.

2.2.5.3 *Chondrogenesis*

Chondrogenic differentiation is carried out in pellet cultures for a period of three weeks. Quadruplicate pellets were formed for each culture at a density of 250,000 cells / pellet in screw-capped 1.5 ml tubes (Nunc) and centrifuged at 100 x g for 5 minutes. Human MSCs were suspended in incomplete chondrogenic medium (ICM) (DMEM high glucose; 100 nM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate; 40 μg/ml L-proline; 1% insulin, transferrin, selenous acid (ITS) supplement; 1 mM sodium pyruvate (Gibco); 1% penicillin/streptomycin) and then re-centrifuged. The cell pellet was then resuspended in complete chondrogenic medium (CCM) (ICM with 10 ng/ml Transforming Growth Factor Beta 3 (TGF-β3; R&D Systems), at 0.5 ml/pellet and were re-centrifuged as described above. The screw caps were loosened to allow gaseous exchange and the cells were incubated under normal growth conditions at 37°C, 5% CO₂ and 90% humidity. Medium was changed three times a week for 21 days. The cells were then harvested for quantification of GAG production using the 1, 9-dimethylmethylen blue (DMMB) assay (Barbosa et al., 2003, Panin et al., 1986) or for histology.

2.2.5.3.1 GAG Quantification Assay

The DMMB assay was used to assess the sulfated GAG content of the pellet cultures. The individual pellets were washed in D-PBS and digested in papain digestion buffer (papain in dilution buffer at 25 μg/ml) overnight at 60°C. Chondroitin sulfate standards were prepared using dilution buffer (50 mM sodium phosphate, 2 mM EDTA, 2 mM N-acetyl cysteine, pH 6.5) to achieve concentrations ranging from 0 μg to 2 μg. Standards were measured in technical replicates for each concentration in a 96-well microtitre plate. Pellet digest of each sample was added in duplicate to each well of a 96-well plate, followed by DMMB stock solution (16 mg of DMMB dissolved in 5 ml reagent grade 100% ethanol combined with 2.73 g NaCl, 3.04 g glycine and 0.69 ml of concentrated HCl (11.6 M) in distilled water
adjusted to pH 3 and volume adjusted to 1 litre). Digested pellet cultures were read on a Wallac Victor™ 1420 Multilabel Counter fluorescent plate reader at 595 nm within 5 minutes of adding the DMMB stock solution.

2.2.5.3.2 DNA Quantification Assay

The GAG quantification was normalized to cell number by analysing the DNA content of the pellets. This was assessed using the Quant-iT PicoGreen dsDNA assay kit (Molecular Probes). Briefly, stock solutions were prepared according to the manufacturer’s instructions. Papain digested samples were diluted 1 in 25 in DMMB dilution buffer. One hundred µl (in triplicate) of both samples and standards provided (DNA 100 µg/ml diluted in 200 mM Tris-HCl, 20 mM EDTA, pH 7.5), were added to the wells of a 96-well black flat-bottomed plate, followed by the addition of PicoGreen solution (diluted in Tris-EDTA; TE) and incubated for 3 minutes at room temperature. Samples were excited at 485nm and read at 538nm on a Wallac Victor™ 1420 Multilabel Counter fluorescent plate reader. The combination of DNA concentrations with the GAG measurements allowed expression of the amount of GAG/pellet as a ratio of the amount of DNA/pellet.

2.2.5.3.3 Histological Analysis

Histological analysis of the pellet was carried out after 21 days in culture. The pellets were washed with D-PBS and fixed for 20 minutes in 10% formalin. The pellets were then wrapped in formalin soaked filter paper (Whatman), placed in an embedding cassette and then placed into a LEICA ASP 300 tissue processor (Vashaw Scientific Inc.) overnight. While in the processor, the tissues were fixed in formalin for approximately 6 hours, dehydrated in ethanol solutions of increasing concentrations: 70% ethanol for 15 min, 90% ethanol for 15 minutes, 100% ethanol for 15 minutes twice, 100% ethanol for 30 minutes and 100% ethanol for 45 minutes. Clearing was then carried out to displace the ethanol with 3 changes of xylene for 20 minutes and one for 45 minutes. The tissues were infiltrated by histological wax with three wax changes, two for 30 minutes and one for 45 minutes. The pellets were subsequently paraffin embedded using embedding moulds and embedding cassettes with the LEICA EG 1150H heated paraffin
embedding system (Vashaw Scientific Inc.). The embedded pellets were sectioned into 5 µm sections using a LEICA RM 2235 microtome (Vashaw Scientific Inc.), mounted onto SuperSoft Plus microscope slides (Gerhard-Menzel) and left at 60°C overnight to dry.

The sections were deparaffinised in Histoclear (National Diagnostics) twice for 5 minutes and rehydrated in 100% ethanol twice for 2 minutes followed by 95% and 70% ethanol for 20 seconds. Following rehydration, the slides were washed in water for 1 minute and subsequently stained with Mayer’s hematoxylin for 6 minutes. Sections were washed with water for 5 minutes and placed in 0.02% fast green for 4 minutes, then rinsed with 1% acetic acid for 3 seconds and finally stained with 0.1% Safranin O for 6 minutes to display cartilage sulfated proteoglycan. The slides were put through washes again, twice in 95% ethanol for 1 minute and twice in 100% for 2 minutes, then twice in Histoclear for 2 minutes. Immediately after removing the slides from the Histoclear, they were mounted with Histomount (National Diagnostics) and coverslipped. The sections were imaged using a confocal microscope (Olympus IX71) with imaging software (Olympus cell™P) at magnifications of 40 x and 100 x.

2.2.5.3.4 Collagen II Immunohistochemistry

Briefly, after the sections were heated at 60°C for 1 hour, sequential deparaffinisation was carried out as described above. The sections were rinsed in deionised water for 5 minutes and endogenous peroxidase activity was quenched by treating the sections with 0.3% Hydrogen peroxide (H₂O₂) in methanol (VWR International Ltd.) for 15 minutes. After the sections were rinsed in deionised water and tris buffer solution (TBS; 0.05 M trizma base, 0.15 M NaCl, pH 7.6) twice, endogenous chondroitin sulfate was digested by 40 mU/ml chondroitinase ABC in 0.1M tris/acetate pH 7.6 containing 1% Bovine Serum Albumin (BSA) for 30 minutes at 37°C. The sections were washed twice for 5 minutes with TBS and blocked with goat serum (KPL Labs) for 1 hour in a humidity chamber followed by incubation with the primary antibody overnight (Collagen II antibody (Abcam) was applied directly without dilution). The sections were treated with a goat anti-mouse
secondary antibody (KPL), followed by streptavidin-biotin (KPL Kit) treatment for 1 hour each and then washed twice. 3,3’-Diaminobenzidine (DAB, Zymed Laboratories Inc.) was added to sections for 10 minutes for brown colour development and washed twice, after which Mayer’s hematoxylin counterstaining was applied for 20 seconds. Slides were washed in water followed by dehydration through graded ethanols and Histoclear as described above. Slides were coverslipped using Histomount and imaged using a confocal microscope (Olympus IX71) with imaging software (Olympus cell^P).

2.2.6 Colony Forming Unit Fibroblasts (CFU-Fs)

Freshly isolated BM was washed with PBS and centrifuged at 900 x g for 10 minutes. The pellet was then resuspended in complete hMSC medium. The BM (100 µl) was added to 10 ml of hMSC medium supplemented with 5 ng/ml FGF-2 (R&D Systems) and seeded onto 100 mm sterile HA, CS or HS-coated culture dishes (Nunc). For the dishes with the EA cells, the unbound BM was removed and the dish was washed twice with 5 ml of serum-free / supplement-free αMEM medium 24 hours after plating. For the LA cell populations, this was carried out after 5 days; the unbound BM was washed off as above. The bound cells were then left to expand for 14 days, after which the colonies were fixed in 10% formalin for 20 minutes, washed with D-PBS and stained with crystal violet solution (2.3% w/v) for 10 seconds. The dishes were then washed with water until it ran clear. A grid was drawn beneath the dish to aid the counting of the colonies. Colonies were manually counted and the results were displayed as CFU-F/10^5 MSCs.
2.2.7 Cumulative Population Doublings

Total population doublings (PD), the total number of times the MSCs have doubled after seeding, was calculated. Human MSCs were cultured from primary (BMMNC) to passage 5. Following each passage the population doublings were calculated based on the initial number plated and the number of cells harvested versus the duration of culture time (in days). To account for differences in actual numbers of adherent cells present in the initial BMMNC cultures, the initial number of cells seeded was set as the number of CFU-F formed (i.e., if 10 CFU-F were formed, then 10 was set as the number of cells seeded). The following formula to calculate PDs was applied:

\[ PD = \frac{\ln(2) \left( \frac{N_{\text{harvest}}}{N_{\text{Initial}}} \right)}{\ln(2)} \]

\( N_{\text{initial}} \) = CFUs formed following 14 days of initial primary culture and the number plated for each subsequent passage.

\( N_{\text{harvest}} \) = number of MSCs harvested at confluence.

2.2.8 Analysis for Cell Surface Expression of MSC Markers

Cells were analyzed for cell surface marker expression by flow cytometry using the ExpressPlus software on the Guava Cytosoft instrument (Guava Technologies). Antibodies are listed in Table 2.1. All antibodies were labelled with phycoerythrin (PE).

2.2.8.1 Guava Cytosoft Analysis:

To determine if expression of traditional MSC proteins is maintained in cell populations, MSCs were trypsinized (Section 2.2.2) and resuspended in staining
buffer (PBS, 2% FBS and 1 mM EDTA - Miltenyi). The cell suspension was centrifuged at 400 x g for 5 min, resuspended in autoMACS rinsing solution, and seeded, in duplicate, at 1 x 10^5 cells/well (3.1x10^5/cm^2) in a 96-well round-bottom plate. Samples were incubated, on ice, for 30 min followed by centrifugation at 500 x g for 5 minutes. The supernatant was then removed and the pellet again resuspended in rinsing solution and centrifugation repeated twice. Following the final removal of supernatant, the MSCs were incubated, on ice, with the antibody of interest for 30 minutes. The MSCs were centrifuged as before and the supernatant was aspirated followed by pellet washing three times in MACS staining buffer. The buffer was carefully removed and MSCs were resuspended in serum-free medium before analysis using the ExpressPlus software on the Guava Cytosoft instrument (Guava Technologies). The number of cells expressing the marker was determined verses control unstained cells and expressed as a percentage of the total population. Controls included cells alone (no antibody) and cells incubated with a mouse anti-human IgG1 isotype control.

<table>
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<tr>
<th>Antibody</th>
<th>Dilutions</th>
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2.2.8.1 Differential Expression of CD44 Receptor between Populations

Mean fluorescence intensity (MFI) was used as a surrogate measure of receptor expression levels. This was calculated from the fluorescent intensity of bound antibody to CD44 was recorded and plotted as log of signal intensity.

2.2.9 CS/HA Exposure at Passage 1 on Pre-Isolated MSCs

MSCs isolated as described in section 2.2.1 were thawed from frozen. These P0 cells had been cryopreserved at -150° and thawed in serum containing medium at room temperature. The cells were then plated in CS or HA-coated plates at 6 x 10³ hMSC/cm². Once confluent, the MSCs were passaged as described in section 2.2.2 above onto non-ECM-coated plates, cultured to the end of P2 and assessed for chondrogenic differentiation using DMMB and Picogreen assays as described above.

2.2.10 Gene Expression Analysis

2.2.10.1 Isolation of RNA from cultured cells

Ribonucleic acid (RNA) isolation from adherent cells was performed using Trizol reagent (Invitrogen), as per manufacturer’s instructions. For adherent cells, following the removal of culture medium, 1 ml of Trizol reagent was added to each well (9.6 cm²) and pipetted over the cells several times. The cell lysate was collected into a 1.5 ml Eppendorf tube and stored at -80°C until required. When required, the samples were thawed and allowed to reach room temperature for 5 minutes. Chloroform (200 µl) was added to the cell lysate, mixed by shaking and incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes at 4°C to separate the solution into two phases. The upper RNA-containing aqueous phase was carefully transferred to a fresh 1.5 ml Eppendorf taking care to avoid the interphase material. The RNA was precipitated from this solution by adding 500 µl of 100% isopropanol, mixing and incubating at
room temperature for 10 minutes. Samples were centrifuged at 12,000 x g for 10 min to pellet the RNA. The supernatant was removed and the RNA pellet washed with 1 ml 75% ethanol. After centrifugation, the supernatant was removed and the pellet air-dried for 5-10 minutes. The RNA was dissolved in 20-40 µl of RNase-free water and then incubated at 55°C for 10 minutes using a heating block.

The concentration and purity of the RNA was determined using the Nanodrop ND-1000 (Nanodrop Technologies). Samples with an A260/A280 ratio of RNA <1.7 were discarded. RNA was diluted with RNase-free water to the desired concentration and stored at -80°C until required for further experimentation.

2.2.10.2 Isolation of RNA from Chondrogenic Pellets

Four replicate chondrogenic pellets were pooled and dissociated using a TissueRupter (Qiagen) prior to purification of RNA as described in Section 2.2.11.1

2.2.10.3 PCR Analysis for SOX9 Transcription

The samples of interest were assessed for relative transcript levels using real-time RT-PCR. RNA (50-100 ng of each sample in 3 µl final volume) was combined with SOX9 primers to a final concentration of 0.5 µM and amplified using the Qiagen Quantitect® Sybr® Green RT-PCR kit (Qiagen; Table 2.3). The level of 18S transcript was used as a normaliser. The amplification conditions were as follows: 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of: 94°C for 15 sec and 60°C for 1 minutes (data was collected at the end of this step). A dissociation (melt) curve was run to verify there were no contaminating products present in the reaction.

Relative gene expression was analysed using the 2-ΔΔct method (Livak and Schmittgen, 2001, Pfaffl, 2001). The average Ct was calculated for the gene of interest and for the normalizing gene. The ΔCT (Ct gene of interest – Ct normaliser) was calculated. From this the 2-ΔΔct could be determined and the levels of gene expression calculated compared to control cells.
The SOX9 primer sequence was 5’-CATGAGCGAGGGCActCC-3’ and 5’-TCGCTTCAGGTCAGCCTTG-3’. The endogenous control 18S rRNA primer sequence was 5’-GTAACCCGTGAAACCATT-3’ and 5’-CCATCCAATCGGTAGTAGCG-3’.

Table 2.2 RT-PCR mix components

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<td>QuantiTect RT Mix</td>
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2.2.11 Statistical Analysis

All values are presented as the mean ± standard deviation of the mean (SD). Data sets were tested for significance using the One-Way ANOVA (non-parametric) and Tukey post-test to compare between groups. A level of p ≤ 0.01 or 0.05 was considered statistically significant. These statistical analyses were used unless otherwise stated.
2.3 Results

2.3.1 Selection of optimal ECM components for selection of chondroprogenitors

Cells within the joint interact with and are influenced by the surrounding ECM via receptors present on their surfaces (Hynes et al., 1992). To test the hypothesis that a sub-population of chondroprogenitors could be isolated from bone marrow via adhesion to ECM molecules, tissue culture plastic was coated using 1 μg/ml cartilaginous matrix molecules as described in section 2.2.3. Two HA preparations were used; Durolane® (Smith & Nephew), which contains a highly purified form of HA produced by bacterial fermentation and is suspended in PBS at a concentration of 20 mg/ml, and Supartz® which is a highly purified sodium hyaluronate that is extracted from rooster combs is provided at a concentration of 10 mg/ml. Heparan Sulfate (a GAG which is isolated from bovine kidney) and CS (isolated from shark cartilage).

Furthermore, it was hypothesised that isolation of MSC sub-populations could be achieved by selective adherence to ECM components. Adherent BMMNC with the potential to rapidly bind to the surfaces were selected 24 hours after plating. These early adherent (EA) cultures were compared to late adherent (LA) cultures where CFU-Fs were allowed to attach for 5 days before removal of residual marrow. All selected populations and the parent population, cultured on non-coated plastic with BMMNCs allowed to attach directly to the tissue culture surface for 1 day (Plastic EA) or 5 days (Plastic LA), were assessed for chondrogenic capacity at P3 as described in Figure 2.2. Durolane (HA 1) EA and CS EA isolated MSCs produced significantly more GAG compared to the MSCs isolated on the other ECM molecules and the control non-coated plastic MSCs (Figure 2.3). These optimal formulations were selected for subsequent experiments with HA 1 (Durolane, referred to as HA) and CS-coated plates.
Figure 2.3: Chondrogenic differentiation of MSCs isolated via adhesion to ECM-coated flasks: Graphical representation of sulphated-GAG production normalized to DNA content after 21 days chondrogenic differentiation of EA and LA MSCs isolated via adhesion to various cartilage ECM molecules. EA populations selected by adhesion to Durolane HA or CS showed significantly higher levels of GAG/DNA compared to all other groups. Results are presented as the mean ± SD of 3 technical replicates from 1 donor, * = p ≤ 0.0001.
2.3.2 Colony Forming Ability of ECM Isolated Populations

One of the criteria to define an MSC includes the ability of the heterogeneous, adherent population in the marrow to form colonies. The colony forming ability of fibroblastic-like cells is measured as CFU-F. Each colony is derived from one cell (Horwitz, Le, Dominici et al., 2005). Anything from 10-15 cells can be defined as a colony. This assay was carried out to determine if the colony forming abilities of the ECM isolated populations were altered. Results displaying equal ability within all populations would establish that colony forming units were not altered. However the CFU-Fs are heterogeneous populations so a lower number of CFU-Fs present in the HA/CS populations would perhaps suggest a more homogeneous population. Figure 2.4 shows each population of isolated MSCs tested for their individual presence of colony forming cells. There was a consistent trend of lower numbers of CFU-F in early populations of the CS and HA coated dishes. However, with the compiled data from all donors there was no statistical significance between groups (Figure 2.4 A). Macroscopic images were taken of the flasks containing the CFU-Fs stained with Crystal Violet to enable counting of the colonies (B).
Figure 2.4: Colony Forming Unit Fibroblasts: (A) Graphical presentation of the CFU-F of ECM isolated MSC populations. Data is presented as fold change from plastic-adherent cultures. The CFU-F number did not vary significantly between isolation methods (p>0.05). Results are presented as the mean ± SD of 4 donors. (B) Macroscopic images of crystal violet stained CFU-Fs.
**2.3.3 Morphological Characteristics of MSC Populations**

Images were taken of each of the treatment groups at P1 to determine whether the isolated populations retained the normal fibroblastic morphology of MSCs. As shown in **Figure 2.5**, the populations of cells all retained the characteristic fibroblast-like morphology (Caplan et al., 1991). Additionally, all populations of cells had similar morphologies to each other.

**Figure 2.5: Morphological Characterisation of Isolated Marrow Stromal Cells**: Phase contrast micrographs showing cell morphology 10 days after initial plating. All populations had a fibroblastic morphology with long, thin cell processes characteristic of MSCs. Scale bar = 200 μm.
2.3.4 Cumulative Population Doubling Analysis

All MSC populations were cultured over 28 days to determine whether the ECM isolated cell’s proliferation capacity was altered. There were no significant differences in proliferation capacity of each MSC population after 2 passages (p>0.05) but in extended culture the population isolated using HA LA showed an increased proliferation rate compared to the other groups (p<0.05) (Figure 2.6). HA has been shown previously to enhance proliferation of various cell types (Kawasaki et al., 1999 and Ahrens et al., 2001).

Figure 2.6: Cumulative Population Doublings of ECM isolated EA and LA cells: Graphical representation of population doublings, from initial primary isolation and subsequently over 3 passages. All cell populations had similar doublings until approximately day 22 where HA LA demonstrated significantly greater growth rates. Results are presented as the mean ± SD of 6 donors. * indicates p≤0.01.
2.3.5 Cell Surface Analysis by Flow Cytometry, for MSC Markers in all Populations

In order to determine the characteristics of the cell populations and to compare the populations to each other, flow cytometry analysis was carried out using antibodies for typical MSC cell surface receptors (Horwitz, Le, Dominici et al. 2005). The standard MSC marker antibodies that were used to carry out surface marker analysis were CD105, CD73, CD44 and CD90. Following cytometric analysis of the cells, it was determined that each of the HA populations and each of the CS populations had very similar surface characteristics to the control, traditionally isolated cells i.e. there was over 97% positive cells in each population for CD105, CD73, CD44 and CD90 and negative for CD45 and CD34 (Table 2.3 A, B).

Table 2.3: Cell surface phenotype analysis of ECM-isolated EA and LA cells: (A) MSC populations were analysed at end of passage 1 for expression of positive MSC cell surface receptors CD105, CD73, CD44 and CD90 by flow cytometry. All ECM isolated MSC populations expressed MSC surface markers (>97%).
Table 2.3: Cell surface phenotype analysis of ECM-isolated EA and LA cells: (B) The non-MSC/hematopoietic markers, CD34 and CD45 were also analysed and were shown to be negative in all populations (<1%). Values are presented as the mean ± SD of 4 donors.

<table>
<thead>
<tr>
<th>Surface Marker Expression</th>
<th>CD34</th>
<th>CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic EA</td>
<td>0.57% ± 0.07</td>
<td>0.09% ± 0.02</td>
</tr>
<tr>
<td>Plastic LA</td>
<td>0.70% ± 0.04</td>
<td>0.12% ± 0.04</td>
</tr>
<tr>
<td>HA EA</td>
<td>0.71% ± 0.02</td>
<td>0.14% ± 0.07</td>
</tr>
<tr>
<td>HA LA</td>
<td>0.83% ± 0.03</td>
<td>0.16% ± 0.06</td>
</tr>
<tr>
<td>CS EA</td>
<td>0.86% ± 0.07</td>
<td>0.09% ± 0.04</td>
</tr>
<tr>
<td>CS LA</td>
<td>0.92% ± 0.09</td>
<td>0.15% ± 0.09</td>
</tr>
</tbody>
</table>
2.3.6 Adipogenic Differentiation of ECM-Isolated Populations

The ability of all ECM hMSC populations to retain their multipotentiality is important for their efficacy. The plastic, HA and CS isolated cells, both early adherent (EA) and late adherent (LA) populations were assessed after two passages for their multipotentiality. The potential of the cell populations to differentiate into adipocytes was assessed after exposure to adipogenic conditions (Figure 2.7, A, B).

Oil Red O staining of intracellular lipid vacuoles identified adipocytes in induced adipogenic cultures of all hMSC populations (A. i-iii, v-vii) while control undifferentiated hMSCs stained negative (A. iv).

Adipogenesis was quantified through Oil Red O extraction from the adipocytes and demonstrated no significant changes in adipogenic potential between any of the MSC populations. All populations retained their potential for adipogenic differentiation (Figure 2.7, B).

**Figure 2.7: Characterisation of the Adipogenic Differentiation Potential of ECM isolated MSC populations:** (A) Light microscopy analysis of all treated MSC populations was positive for Oil Red O staining of the lipid vesicles while the undifferentiated control (A. iv) was negative. Scale bar = 200 µm.
Figure 2.7: Characterisation of the Adipogenic Differentiation Potential of ECM isolated MSC populations: (B) Quantification of Oil Red O extraction from MSC populations indicated no significant difference in adipogenic potential between groups (p>0.1). Data is presented as fold change from plastic-adherent cultures. Values are presented as the mean ± SD from 4 donors.
2.3.7 Osteogenic Differentiation of ECM-Isolated Populations

The plastic, HA and CS isolated cells, both early adherent (EA) and late adherent (LA) populations were assessed after two passages for their osteogenic potential (Figure 2.8, A, B).

Alizarin Red staining revealed enhanced matrix deposition in osteogenically induced hMSCs (A. i-iii, v-vii) in comparison to undifferentiated control wells (A. iv). Calcium deposition of osteogenically treated MSC populations also demonstrated the ability to retain osteogenic differentiation while revealing no significant difference between each group (Figure 2.8, B).

Figure 2.8: Characterisation of the Osteogenic Differentiation Potential of ECM isolated MSC populations: (A) Light microscopy analysis of MSC populations showed positive staining by Alizarin Red of osteocyte matrix deposits. The undifferentiated control (A, iv.) was shown to be negative for matrix staining. Scale bar = 500 μm.
Figure 2.8: Characterisation of the Osteogenic Differentiation Potential of ECM isolated MSC populations: (B) Quantification of calcium deposition by the cells demonstrated no significant difference in osteogenic differentiation potential between groups (p>0.1). Data is presented as fold change from plastic-adherent cultures Values are presented as the mean ± SD from 4 donors.


2.3.8 Chondrogenic Differentiation of ECM-Isolated MSCs

Chondrogenic differentiation was carried out to determine if ECM molecules can be used to isolate a mesenchymal progenitor cell for enhanced cartilage production. Differentiation was assessed in all populations of MSCs after they were pelleted and treated with CCM for 21 days of culture (Figure 2.9, A). The gold standard for chondrogenic differentiation is production of GAG normalised to DNA content and the histological analysis of pellets which are can be treated with Safranin O, which stains cartilaginous proteoglycans a pink/red colour. Chondrogenic pellets were histologically analysed and results demonstrated an increase in pellet size and staining due to the increased production of GAG (A, i-iii, v-vii). GAG was visualised by staining with Safranin O which stains proteoglycans with a pink/red colour. The blue colour shows a negative result for the presence of proteoglycans. Despite all treated groups staining positively for proteoglycans, the HA EA and CS EA pellets both stained more intensively for Safranin-O and pellets were larger than the other MSC groups (A, ii, iii). Magnified images of these pellets are shown to demonstrate the large amount of proteoglycan produced by the cells. The HA treated pellet displayed more GAG throughout, with more positive stain towards the outside than the other treatment groups (A, ii). The negative pellet demonstrated a malformed pellet completely negative for Safranin-O staining (A, iv). Chondrogenesis was quantified for GAG per DNA content and a significant increase was observed in HA EA and CS EA compared to P EA, P LA and HA LA. The highest levels of GAG were produced in the HA EA population. Following this was the CS EA population which also had significantly higher levels of GAG indicating that the population of cells isolated with ECM molecules were more capable of differentiating into chondrocytes. The differentiation results were consistent with the previous results in Figure 2.3.

2.3.8.1 Wet Weight of Chondrogenic Pellets
**Figure 2.9, C** demonstrated the wet weight of the various ECM isolated MSC chondrogenic pellets after 21 days in culture. There was a significant increase in weight in the HA EA and CS EA populations and this increase correlated with the GAG production observed in Figure 2.9 A and B.

*Figure 2.9: Characterisation of the Chondrogenic Differentiation Potential of ECM-Isolated MSC populations:* (A) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans produced by chondrocytes. Sulfated GAG was present in all ECM isolated MSC populations. The undifferentiated control (A, iv) was shown to be negative for both the Safranin O stain and the rounded phenotype of positive chondrogenic pellets. Scale bar = 500 μm.
Figure 2.9: Characterisation of the Chondrogenic Differentiation Potential of ECM-Isolated MSC populations: (B) GAG per DNA content was quantified for all ECM isolated MSC populations. There were significant increases in the chondrogenic differentiation of HA EA and CS EA isolated populations ($p\leq0.01$) versus all other groups except the CS LA group. * indicates $p\leq0.01$; Data is presented as fold change from plastic-adherent cultures. Values are presented as the mean ± SD from 4 donors.
Figure 2.9: Characterisation of the Chondrogenic Differentiation Potential of ECM-Isoleted MSC populations: (C) Wet Weight of Chondrogenic Pellets. Graphical representation of pellet wet weight after 21 days in chondrogenic culture. There was a significant increase in pellet wet weight in the HA EA and CS EA MSC populations compared to all other groups (p≤0.01). * indicate p≤0.01; Values are presented as the mean ± standard SD from 3 donors.
2.3.9 Analysis for the Presence of Residual GAG Molecules from MSC-Isolation

GAG production is measured using a chondroitin sulfate based assay. Therefore, it was necessary to rule out that the significant increase in GAG production seen after ECM-mediated cell isolation was not due to the presence of remaining HA/CS used for culture surface coating. Chondrogenic analysis was carried out in both CCM and ICM, lacking TGF-β3. The presence of GAG in the ICM treated cells would indicate residual coating molecules present. The results demonstrated negligible GAG levels present in the ICM, non-chondrogenically primed pellets.

These results validated that there were no GAG molecules remaining on the ECM isolated cells and GAG levels detected in the treated pellets were due to synthesised GAG produced by CCM-treated pellets (Figure 2.10). The histologically treated pellets validated the negative chondrogenic differentiation, showing that a rounded chondrocytic phenotype and staining for proteoglycans is lacking. Images of two of the sections, one CCM treated and one ICM treated were enlarged in order to highlight the differences between the differentiated and un-differentiated pellets.
**Figure 2.10: Presence of Residual GAG from Isolation Stage:** HA and CS isolated MSCs underwent chondrogenic differentiation with complete (CCM) and incomplete chondrogenic medium (ICM - no TGF-β3). (A) The absence of TGF-β3 resulted in the HA (ICM) and CS (ICM) producing negligible GAG as compared to the CCM treated pellets. * indicates p<0.0001; Results are presented as the mean ± SD from 3 donors.
**Figure 2.10: Presence of Residual GAG from Isolation Stage:** (B) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans produced by the chondrocytes. Sulfated GAG was present in all CCM treated pellets. The ICM treated pellets were shown to be undifferentiated and were negative for both the Safranin O stain and the rounded phenotype of positive chondrogenic pellets. Scale bar = 500 μm.
2.3.10 Differential Expression of CD44 Receptor between Populations

CD44 is the main receptor for both HA and CS expressed by cells (Aruffo et al., 1990). The mean florescent intensity of the MSC surface receptor CD44 was analysed to assess any changes in the expression levels of the receptor after ECM molecule exposure and subsequent culture. There were no significant changes in CD44 expression levels on the cell surface of MSCs isolated using HA or CS compared to traditionally isolated methods (Figure 2.11).

![Figure 2.11: Cell surface expression levels of CD44 on ECM isolated MSCs](image)

Figure 2.11: Cell surface expression levels of CD44 on ECM isolated MSCs: There was no statistical difference in the cell surface expression levels of CD44 between ECM isolated groups (p>0.05). Results are presented as the mean ± SD of 3 donors.
2.3.11 Exposure of Cells to CS at the End of Passage 1

In order to confirm that the CS effect on the chondrogenic ability of the MSCs was a phenomenon only seen upon isolation from BM, traditionally isolated MSCs were exposed to CS at passage 1 and then expanded through passage 2 on non-ECM coated plates before chondrogenic induction. There was no significant increase in chondrogenic potential by adding passage 1 MSCs to CS-coated plates (Figure 2.12, A, B). This data demonstrated that CS exposure did not have a positive effect on the chondrogenic ability of traditionally isolated MSCs at passage 1.

Figure 2.12: CS Exposure in Passage 1 on Traditionally Isolated MSCs: Chondrogenic differentiation capacity was measured in traditionally isolated MSCs and MSCs that were traditionally isolated but which were exposed to CS at passage 1. (A) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans and immune-stained for collagen type II produced by the chondrocytes. Sulfated GAG and collagen type II were detected in both treatments. Scale bar = 500 μm.
Figure 2.12: *CS Exposure in Passage 1 on Traditionally Isolated MSCs: (B)*
Glycosaminoglycan per DNA content was quantified for both populations at 21 days. There was no significant change in chondrogenic ability in the CS exposed cells $p>0.05$. Results are presented as the mean ± SD of 3 donors.
2.3.12 Exposure of Cells to HA at the End of Passage 1

As in section 2.3.11, in order to confirm that the HA effect on the chondrogenic ability of the MSCs was a phenomenon only seen upon isolation from BM, traditionally isolated MSCs were exposed to HA at passage 1 and then expanded through passage 2 on non-ECM coated plates before chondrogenic induction. The addition of passage 1 MSCs to HA-coated plates did not result in any significant changes in chondrogenesis (Figure 2.13, A, B). This data demonstrates that HA exposure did not have a positive effect on the chondrogenic ability of traditionally isolated MSCs at passage 1.

Figure 2.13: HA Exposure in Passage 1 on Traditionally Isolated MSCs: Chondrogenic differentiation capacity was measured in traditionally isolated MSCs and MSCs that were traditionally isolated but which were exposed to HA at passage 1. (A) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans produced by the chondrocytes. Sulfated GAG was detected in both treatments. Scale bar = 500 μm.
Figure 2.13: HA Exposure in Passage 1 on Traditionally Isolated MSCs (B) Glycosaminoglycan per DNA content was quantified for both populations at 21 days. There was no significant change in chondrogenic ability in the HA exposed cells, p>0.05. Results are presented as the mean ± SD of 3 donors.
2.3.13 PCR analysis of SOX9 Expression in P EA, HA EA and CS EA MSC Populations

PCR was carried out to analyse SOX9 expression in the chondrogenically primed pellets. SOX9 is the main transcription factor associated with chondrogenesis which peaks at around 24 hours after chondrogenic induction (Akiyama et al., 2002). This assessment was carried out using qPCR and analysed using the \( \Delta \Delta CT \) Method, where 18S acted as the endogenous control and P EA T0 acted as the calibrator. The cells were pelleted in CCM for 3 minutes [Day 0 (D0)], for 24 hours [Day 1 (D1)], 4 days (D4) and 7 days (D7) to measure the temporal expression of SOX9. Expression was evident in the P early adherent (EA), HA EA and CS EA populations. HA EA MSCs were shown to have significantly higher levels of SOX9 than all other time-points and treatment groups. The expression of SOX9 dropped after 24 hours in all groups, which is expected. However the transcription of SOX9 remained significantly higher in HA EA D4 and D7 compared to the other groups at these time-points (Figure 2.14).
Figure 2.14: SOX9 gene expression: PCR analysis was carried out on the HA EA and CS EA populations and the P EA control population to analyse SOX9 transcription at 0 hours, 24 hours, 4 days and 7 days in chondrogenic differentiation conditions. HA EA D1 chondrogenic pellets were shown to have significantly higher levels of SOX9 compared to all other time-points and treatments ($p \leq 0.0001$). SOX9 expression was shown to remain at significantly higher levels in HA EA D4 than HA EA D7 ($p \leq 0.01$) compared to P and CS isolation at both time-points.* = $p \leq 0.001$ (same time-points); ** = $p \leq 0.0001$ (all groups). Results are presented as the mean ± SD of 3 donors.
2.4 Discussion

MSCs have emerged as a cell source that has the potential for inclusion in cellular therapies for articular cartilage repair. The advantages of MSCs include their availability in multiple tissue sources and ease of isolation, their capacity for proliferation, multipotent differentiation and specifically their ability to undergo chondrogenic differentiation in the presence of chondrogenic supplements (Pittenger et al., 1999). MSCs have been isolated from a number of sources as outlined in the introduction (Section 1.4). In relation to the joint, stem cells have been isolated from the infrapatellar fat pad, the synovium, synovial fluid and the periosteum (English et al 2007; De Bari et al., 2001; Jones, 2008; Buckley et al., 2010). Bone marrow stem cells have been shown to be reliably isolated and cultured. Clonal analysis of these cells has described how 20-50% of these cells could be truly tri-potent. More importantly, this analysis has also identified a small sub-population of cells which are solely chondrogenic or, chondroprogenitors (Pittenger et al., 1999). Consequently, MSCs possess practical advantages that have potential for articular cartilage repair in OA patients. This study has elucidated a process that enables a putative sub-set of chondroprogenitor cells to be isolated from human bone marrow in vitro.

This present study looked at the ability of a number of cartilaginous ECM molecules to isolate a sub-population of progenitors from the marrow by coating on tissue culture plastic and subsequently being bound by the BM MSCs. Collagen type II, which is critical in chondrogenesis was not analysed here as molecules involved in early commitment were chosen. Two molecules were initially identified to promote the isolation of MSCs with an increase in chondrogenic potential directly from the marrow. These molecules were Durolane hyaluronic acid and chondroitin-6-sulfate. These polysaccharides demonstrated an ability to isolate MSCs with significantly higher chondrogenic ability as compared with traditionally isolated controls and all other ECM isolated MSCs. The MSCs with enhanced chondrogenic capacity were not only those isolated by HA and CS but more specifically those which adhered to
HA and CS within the first 24 hours of BM plating. As previously stated, Pittenger et al described how individual adherent BM hMSCs were present after 1 day in culture. An observation was made that some colonies displayed limited or more specific differentiation potential. One of their interpretations was that some of these cells might represent progenitor cells, with restricted differentiation potential (Pittenger et al., 1999). We therefore deemed it necessary to also test for these types of early adherent colonies in this study along with the usual technique of allowing BM MSCs to adhere and form colonies for five days before removing non-adhered cells and excess BM constituents. It appears in our study that the chondroprogenitors adhered more readily to the HA/CS than plastic and as a consequence were adhered within the first 24 hours of culture. The five day time-point may have hindered the progenitor’s ability to thrive due to excess competition or high levels of metabolic waste.

The reason for HA and CS being the sole two ECM molecules to have an effect may be that they have a more important role in eliciting differentiation in vivo than the other molecules tested. HA is a major component of the ECM and its presence has been shown to create highways for cell migration (Toole, 2001). A striking example of this effect is shown with the migration of MSCs into the cornea following an increase in HA deposition and hydration (Toole & Trelstad, 1971). It is well documented that HA and CS are important in regulating cell differentiation and recent discoveries have underscored the importance of the physical and chemical characteristics of the matrices in determining stem cell fate (Toole, 1997; Lutolf, 2009; Chen et al., 2011). Addition of HA to synovial fibroblasts has been shown to have major physiological effects by increasing the synthesis of HA (Smith and Ghosh, 1987), CS and other cartilage proteoglycans (Kawasaki et al., 1999; Frean, Abraham, Lees, 1999).

HA’s function or effect can differ slightly due to variations in its in vitro formulations. It can differ in surface characteristics, mode of preparation, HA content, and extent of crosslinking. Durolane contains a large amount of concentrated HA which mimics the effects of natural HA. The patented NASHA
hyaluronic acid stabilisation process enables Durolane to resist breakdown by considerably extending its half-life (NASHA is a natural, stable hyaluronan product which is a stabilised form of HA www.durolane.com). This could explain why Durolane HA had more of a positive effect on the MSCs as opposed to Supartz™ HA (HA Type B) which is a less viscous liquid and more quickly broken down, due to a shorter half-life.

With the evident differences in the HA and CS cells compared to plastic adherent cells shown in Figure 2.3, it was important to assess whether cells isolated by adherence to Durolane HA and CS retained typical MSC characteristics such as MSC cell surface markers, population doublings, MSC phenotype and colony forming ability. These MSC characteristics were demonstrated to be present (figures 2.4-2.9 and Table 2.3). These results demonstrated that MSCs isolated using ECM molecules as early adherent or late adherent cells had the characteristic MSC cell surface receptors present with all populations over 96% positive for all markers. The cells were shown to have typical MSC population doublings. This was concluded using the CFU-F data from Figure 2.4 and the following cumulative population doubling numbers. All populations retained similar doublings during the first two passages. In summary, all MSC populations isolated by adherence to HA/CS retained MSC phenotype and retained considerable colony forming ability. Taken together, these results enable the matrix isolated cells to remain defined as MSCs.

The investigation and development of MSC isolation methods has proven to be challenging due to the distinct lack of specific markers displayed by MCSs in vitro (Jo et al., 2007). With intense investigation, studies have validated methods varying from negative selection, where cell types such as hematopoietic cells are removed (Baddoo et al., 2003), to positive selection, when MSCs are enriched from a pool of cells in which they are known to be present. Molecules have been discovered that could prove useful in the in vivo identification and purification of MSC-like cells. One of these is a neural marker called LNGFR (CD271), repeatedly found expressed on MSCs in vivo (Jones et al., 2002; Jones et al., 2006; Bühring et al., 2007; Gindraux
et al., 2007 and Battula et al 2009) and another is CD49a (Deschaseaux et al., 2003; Jones et al., 2006).

To address the lack of specific markers Buhring et al carried out a screen for specific BM MSC surface markers using monoclonal antibodies (mAB) specific for immature cells. This screen resulted in the selection of 15 novel, MSC-specific markers which selected for clonogenic and morphologically typical MSCs (Buhring et al., 2007). The study has provided novel and improved tools for the selection of MSCs using W8B2, W3D5, W5C5 and 39D5 antibodies. The antibodies provide a combination of the favourable features of selective recognition and bright staining of the CD271 bright cells. 39D5 further recognizes an epitope on CD56 (which plays a role in embryogenesis, development and contact-mediated interactions between neural cells) which is not present on natural killer cells but on a fraction of highly clonogenic CD271 bright cells. However, it was concluded that even narrowly defined, selected MSC clones are highly heterogeneous with respect to their proliferation capacity, their phenotype and their differentiation potential (Battula et al., 2009). An additional concern with antibody-selected MSCs is the rare clonal nature of the populations in marrow; extensive expansion in vitro with many population doublings and possible senescence would be necessary to obtain enough cells for use in therapeutic applications, perhaps hindering the cell’s potential.

There has not been much focus on using biomimetic molecules (i.e. cartilage proteoglycans) to isolate a chondroprogenitor cell. CS and HA have been used with varying results in scaffolds or HA hydrogels loaded with expanded MSCs (Chen et al., 2011; Nettles, 2004; Hahn, et al., 2004; Chung and Burdick, 2009; Matsiko et al., 2012). However, these molecules would appear to have a more selective effect when used as a selection tool for isolating MSCs from primary tissue as seen in this study. It was especially important to assess the tri-lineage potential of the ECM isolated MSCs. If the HA and CS isolated cells did not harbour tri-lineage potential, it would suggest that the ECM isolated cells were true progenitors. However, the cells had the ability for tri-lineage differentiation; adipogenic, osteogenic and chondrogenic differentiation (Figures 2.7-2.9). Nevertheless, this did not rule out
that there is a favouring in selection of chondroprogenitors in the isolation process because there were no significant differences in adipogenic or osteogenic differentiation whereas, the HA and CS early adherent populations demonstrated significantly higher chondrogenic potential than the traditionally isolated plastic controls and the later time-points (Figure 2.9).

The results presented in this chapter differs to other studies using ECM molecules with MSCs in the fact that we are looking at the effect of ECM exposure during the isolation process of MSCs instead of using passaged cells that were isolated traditionally. Other groups have also enlisted the use of growth factors such as BMP-6, BMP-2, BMP-4 and GDF-5 in their chondrogenic differentiation medium in order to increase the differentiation potential of MSCs (Sekiya et al., 2001; Shen et al 2009; Hatakeyama, Tuan and Shum, 2004). One of the studies carried out was to analyse whether CS and HA also had positive effects on traditionally isolated, passage 1 MSCs. There were no significant differences between the CS or HA treated and non-treated passage 1 MSCs (Figure 2.12 and 2.13). It appears that HA and CS have a chondrogenic enhancing effect only upon isolation. This may be due to progenitor cells with ECM specific receptors present in the marrow becoming concentrated and binding rapidly to the HA or CS-coated to the plates. The early isolation of this population and subsequent removal of competing factors after 24 hours in culture may allow this chondrogenic population to thrive and proliferate ensuring a high number of chondroprogenitors in the population.

The outcome measure of chondrogenic potential was shown by a 21 day differentiation assay. This three week time-point is a late event, so this alone cannot conclude that the cells are in fact initially more primed towards chondrogenesis. SOX9, the master transcription factor for chondrogenic differentiation of hMSCs, peaks at approximately 20 hours after initiation of chondrogenesis, which would demonstrate an early event within the HA isolated MSCs. The increased expression of SOX9 seen at Day 1 and remaining at a higher level compared to CS and non-ECM coatings demonstrated that these cells are more primed upon the onset of chondrogenesis (Figure 2.14).
This process of isolating a more homogenous population of progenitors through their selective adherence to ECM molecules may increase the reparative and regenerative capacity of these cells for the treatment of OA and provides cells that are more committed to the chondrogenic lineage.

This work focuses on the cleaner isolation of a homogenous population of MSCs, thus, producing methods that are more applicable in cell therapy. Future work requires the analysis of the marrow components that are removed from the early adherent cells. This fraction may be significantly depleted in chondroprogenitors due to them having adhered to the HA and CS within the first 24 hours.
Chapter 3

Analysis of Non-Adherent Bone Marrow Populations after 1 and 5 day Exposure to Cartilage ECM Molecules and Identification of the most Efficacious ECM Selection Molecule and Time-point.
3.1 Introduction

As chondrocytes regulate the dynamic equilibrium between the production and degradation of the ECM, it is proposed that a loss of chondrocyte viability may result in a predisposition of aged individuals to develop OA (Pennock et al., 2007). The ultimate goal when faced with degeneration in OA is to replace the degenerated cartilage with fully functional articular (hyaline) cartilage. In order to regenerate this articular cartilage the foundation must be based on the type of cells used in therapy and their potential to produce a chondrogenic phenotype. The availability of large quantities of MSCs together with their chondrogenic differentiation capacity after prolonged in vitro expansion have made these cells a promising reparative cell candidate for OA cartilage repair.

MSCs isolated from a BM aspirate represent a very small proportion of the total nucleated cells present (0.001-0.01%). The primary culture period is between 12-14 days. This involves medium changes which deplete the non-adherent cells present in the BM (Pittenger et al., 1999). Based on results from Chapter 2, where it was shown that HA and CS adherent cell populations were enriched in chondroprogenitors, it was hypothesized that the unattached mononuclear cell fraction that is discarded from the early adherent plates was depleted of chondroprogenitors. Conversely, the non-adhered cells remaining suspended in the EA bone marrow require analysis to show a reduction in chondroprogenitor cells to prove this hypothesis. The marrow that was removed from the 5 day plates also needs to be analysed for the presence of any functional late adherent (LA) MSCs left non-adhered.

Adult bone marrow contains both HSCs and MSCs. Since Friedenstein proposed the concept that BM contained precursor cells for mesenchymal cell lineages over 40 years ago (Friedenstein et al., 1968), marrow stromal cells have been characterised based on their properties in vitro or following transplantation into animal models of disease (Kusnetsov et al., 2001). The term CFU-F was coined by Friedenstein to
describe cells that were isolated from the BM stroma; these cells are adherent, fibroblastic and clonogenic in nature (Friedenstein et al., 1974). As this work indicated that CFU-Fs are fibroblastic cells which are highly adherent upon initial plating, it led to a limit in studies into putative alternative phenotypes for BM-resident mesenchymal progenitors.

Despite MSCs having great potential in repair and regeneration in cell therapy and tissue engineering properties (Ciapetti et al., 2006; Brooke et al., 2007; Tseng et al., 2008), one of the main goals in the field still focuses on the optimization of cell isolation and \textit{ex vivo} culture techniques. The impetus for this effort in the case of BM and other tissues is the isolation and functional characterisation of the various populations in the tissues (Neuhuber et al., 2008). Adherence to tissue culture plastic is the oldest and most popular isolation method. Generally cells are given several days to adhere, although there has not been a complete agreement as to the optimal time (Dominici et al., 2006). It is common to allow BMMNC 5 days to adhere (Colter et al., 2000; Jiang et al., 2002; Dominici et al., 2006).

Another issue in translation of MSC therapies is the high cost of marrow and stem cell processing. There is therefore a need to reduce cost by making the final product a more defined and potent population while also maximising yields retrieved from the marrow. Several methods have been described to isolate MSCs from human BM, such as the use of immunomagnetic beads, density gradient separation using Ficoll or Percoll centrifugation as opposed to direct BMMNC plating (Pittenger et al., 1999; Dennis et al., 1992; Jiang et al., 2001; Lennon et al., 2001; Stenderup et al., 2002). Studies are currently being carried out on the safety, feasibility, and efficiency of MSC transplantation for clinical use, and, as several protocols use extremely high numbers of cells (up to 5 million cells/kg body weight), the identification of “optimal” conditions for \textit{in vitro} cell culture requires investigation. Studies being carried such as BM plating using varying plating densities (Mareschi et al., 2011) and using serum free growth conditions (Ankrum and Karp, 2010; Jung et al., 2012).
It has been suggested that non-adherent bone marrow cells have proliferative and differentiative potential similar to adherent cells and may improve yields of MSCs from marrow by utilising more of the MSCs present in different states in the marrow (Wan et al., 2006). However, relatively few papers have been published on the non-adherent or discarded population, but results obtained from various authors suggest that there is an undifferentiated quiescent sub-population of mesenchymal progenitors in the non-adherent BM compartment that may be able to become adherent in vitro, proliferate and differentiate into different tissue lineages (Modder and Khosha 2008; Zhang et al., 2009). Zhang et al initially examined the role of non-adherent MSCs as pluripotent stem cells. It was unclear whether the MSCs resided in BM as adherent fibroblasts or non-adherent round cells, whether they serve as a common stem cell for multiple lineages, whether they are a major source of adult stem cells and whether they migrate through circulation (Zhang et al., 2009). A “pour off” BM culture method was used in their study to determine whether non-adherent BM cells can give rise to CFU-Fs and if they can differentiate into multilineage cells in vitro. This study demonstrated that CFU/MSCs could be derived from the non-adherent fraction of BM and that non-adherent BMSC-derived fibroblastic cells can differentiate into osteocytes, adipocytes and chondrocytes under inducing media in vitro (Zhang et al., 2009).

Differences between the early adherent, late adherent and non-adherent bone marrow stromal cells have not yet been fully elucidated as the non-adherent cells are usually discarded and the EA cells are generally not separated from the LA cells using traditional plastic isolation.

The objective of this chapter was to assess the EA and LA non-adherent populations for putative MSCs and to compare these cells to those isolated by traditional methods and the ECM-isolated populations described in the previous chapter. The second objective was to assess whether the early non-adherent sub-population is depleted in chondroprogenitors due to the increased chondrogenic differentiation in the HA and CS early adherent MSC populations.
3.2 Materials and Methods

All materials were supplied by Sigma Aldrich unless otherwise stated.

3.2.1 ECM Based Isolation of MSCs- Method 2: Non-adherent sub-populations

The BM was processed and the EA and LA populations were isolated as described in Section 2.2.4. To obtain non-adhered mesenchymal sub-populations, the BM non-adherent cells that were removed from the EA culture dishes after 24 hours were replated onto non-ECM coated flasks (Figure 3.1). The BM and its suspended cells were left to reside in this flask for five days to allow adherence of a sufficient number of MSCs. At this point, the adhered cells were washed thoroughly, fresh medium was added and these cells were left to expand to confluence. This population is referred to as the early mesenchymal sub-population (eSP).

To obtain the late mesenchymal sub-population (ISP), the BM and non-adherent cells that were removed from the LA culture dishes after 5 days were replated onto non-ECM coated flasks. The BM and suspended cells were left to reside in this flask for four days to allow adherence of any cells that remained non-adhered after the initial 5 day isolation period culture. After this four day period, the adhered cells were washed thoroughly, fresh medium was added and these cells were left to expand to confluence. This population is referred to as the late mesenchymal sub-population (ISP).
Figure 3.1: Experimental design for isolation of early and late mesenchymal sub-populations: (A) BM was removed after 24 hours from EA flasks to isolate early adherent (EA) cells. The BM was left on the remaining flasks for a further five days allowing for the isolation of late adherent (LA) cells. (B) Non-adhered BMMNCs that were removed from the EA flasks were replated onto non-ECM coated flasks; the population that adhered to these flasks is referred to as the early sub-population (eSP). The BMMNCs that were removed from the LA flasks were also replated onto non-ECM coated flasks; the population that adhered to these flasks is referred to as the late sub-population (ISP). All flasks were expanded to 80% confluence and passaged onto non-coated tissue culture flasks for two passages at the end of P0 before analysis.
Table 3.1 Cell populations: The BMMNCs adhering within the first 24 hours to the non-coated plastic, HA or CS coated plates are referred to as the EA or early adherent populations i.e. P EA, HA EA, CS EA. Replated non-adherent cells after 24 hours are referred to as eSP or the early sub-populations, i.e. PeSP, HAeSP and CSeSP. Cells adhering within 5 days are referred to as the LA or the late adherent populations, i.e. P LA, HA LA, CS LA. Replated, non-adherent cells after 5 days are referred to as ISP or late sub-populations i.e. PISP, HAISP and CSISP.

Analysis of Cell Populations

Analysis of cell populations was carried out as described in materials and methods chapter 2, Sections 2.2.5.1-2.2.5.3 describing adipogenic, osteogenic and chondrogenic differentiation of cell populations. Cumulative population doubling analysis was carried out as described in Section 2.2.7. Analysis for cell surface expression of MSC markers was carried out as in Section 2.2.8. CFU-F analysis was carried out as described in Section 2.2.6. PCR was carried out as described in Section 2.2.10.
3.2.3 Statistical Analysis

All values are presented as the mean ± standard deviation of the mean (SD). Data sets were tested for significance using the One-Way ANOVA (non-parametric) and Tukey post-test to compare between groups. A level of $p \leq 0.01$ or 0.05 was considered statistically significant. These statistical analyses were used unless otherwise stated.
3.3 Results

3.3.1 Morphological Characterisation of ECM Isolated Marrow Stromal Cells and Marrow Sub-Populations

Microscopic analysis was performed for each of the treatment groups at passage 1 to determine whether the early and late sub-populations retained the normal fibroblastic morphology of MSCs. As shown in Figure 3.2, the various populations of cells retained the characteristic fibroblast-like morphology of MSCs (Caplan et al., 1991). Equally, all sub-populations and populations of cells had similar morphologies to each other.

![Figure 3.2: Morphological characterisation of isolated marrow stromal cells and sub-populations](image)

Figure 3.2: Morphological characterisation of isolated marrow stromal cells and sub-populations: Phase contrast micrographs showing cell morphology of all populations 10 days after primary culture. As with the EA and LA populations, all sub-populations retained a fibroblastic morphology with long, thin cell processes characteristic of MSCs and all cells from the various isolation methods had similar cell morphologies. Scale bar = 200 μm
3.3.2 Cumulative Population Doublings of ECM-isolated Populations and Sub-Populations

All MSC populations and sub-populations were cultured over 4 passages in order to determine each sub-population’s proliferation capacity compared to the EA and LA populations. Results are presented as separate graphs to allow for easier demonstration. Apart from the significant increase in HA LA PDs as shown previously in Figure 2.6, there were no other significant differences in proliferation between the EA, LA and eSP MSCs proliferative capacities as shown in Figure 3.3 A and B. There were significant differences in the P, HA and CS-ISP MSCs where their proliferative capacities were significantly lower than the other groups this is due to some cell death once the cells were sub-plated, and subsequent slow proliferation. The lower proliferation is not unexpected as the ISP cells were those remaining suspended in the BM after for 5 days of ECM exposure and the CFU-F were much lower to begin with. The negative PD numbers are due to the fact that there were less cells retrieved from the ISP cultures than were theoretically initially plated (Figure 3.3 B).
Figure 3.3: Cumulative population doublings of ECM isolated EA, LA, eSP and ISP MSC Populations: (A) Growth curves of ECM-isolated populations from initial primary culture and over 3 subsequent passages. All EA and LA cell populations had similar doublings until approximately day 22 where HA LA demonstrated significantly greater population doublings in passage 3. (B) Growth curves of ECM isolated sub-populations demonstrated that the eSPs had similar PDs to the EA and LA Populations. The ISP Populations had significantly slower proliferation rates in the first two passages. * indicates p<0.01. Values are presented as the mean ± SD of data from 3 donors.
3.3.3 Cell Surface Analysis by Flow Cytometry for MSC Markers in ECM-isolated Populations and Sub-Populations

It was necessary to determine more specifically, the characteristics of the sub-populations. In particular, assessment of expression of the typical MSC surface markers was necessary due to the fact that some of these cells were slower to adhere, especially the ISP cells. There was a possibility that this population represented a different population of cells residing in the marrow that can be isolated with re-plating of bone marrow after initial adherence culture. Flow cytometry analysis was carried out at the end of passage 1 using antibodies for typical MSC cell surface receptors (Horwitz, Le, Dominici et al. 2005). The antibodies that were used to carry out surface marker analysis were CD105, CD73, CD44 and CD90. Following cytometric analysis of the cells, all sub-populations were shown to be over 97% positive for all MSC surface markers and negative for the non-MSC markers CD45 and CD34 (Table 3.4 A, B).
**Table 3.2: Cell surface phenotype of ECM isolated eSP and ISP sub-populations:**

(A) MSC populations were analysed at the end of passage 1 for expression of positive MSC cell surface receptors CD105, CD73, CD44 and CD90 by flow cytometry. All ECM isolated MSC populations expressed MSC surface markers (>97%).

(B) The non-MSC/hematopoietic markers, CD34 and CD45 were also analysed and were shown to be negative in all populations (<1%), Values are presented as the mean ± SD of data from 3 donors.
3.3.4 Characterisation of the Adipogenic Differentiation Potential of ECM-isolated Populations and Sub-Populations

It was necessary to analyse whether the sub-populations preserved the ability to differentiate to the same extent as the other MSC populations due to their slower adherent characteristics. Adipogenesis was quantified through Oil Red O extraction from the adipocytes and the sub-population cells demonstrated no significant changes in adipogenic potential compared to the ECM-isolated MSCs. Oil Red O staining of intracellular lipid vacuoles identified adipocytes in induced adipogenic cultures of all MSC populations (Figure 3.4 A. i, ii, iv, v) while control undifferentiated MSCs demonstrated negative staining (A. iii). The sub-populations retained similar potential for adipogenic differentiation to all other groups (B).

![Figure 3.4: Characterisation of the Adipogenic Differentiation Potential of ECM EA and LA isolated MSCs Compared to MSC early and late sub-populations: (A) Representative light microscopy analysis of ECM isolated MSC populations was positive for Oil Red O staining of lipid vesicles while the undifferentiated control was negative for Oil Red O staining. Scale bar = 200 μm (A. iii).](image-url)
Figure 3.4: Characterisation of the Adipogenic Differentiation Potential of ECM EA and LA isolated MSCs Compared to MSC early and late sub-populations: (B) Quantification of Oil Red O extraction from all MSC populations indicated no significant difference in adipogenic potential between any of the ECM-isolated MSC populations and sub-populations (p>0.1). Data is presented as fold change from plastic-adherent cultures. Values are presented as the mean ± SD of data from 3 donors.
3.3.5 Characterisation of the Osteogenic Differentiation Potential of all ECM-isolated Populations and Sub-Populations

Osteogenic differentiation was analysed by assessment of calcium deposition and results indicate successful differentiation as seen with increased calcium levels in MSC sub-populations. Alizarin Red staining revealed enhanced matrix deposition in osteogenically induced hMSCs (Figure 3.5 A. i,ii,iv,v) in comparison to undifferentiated control wells (A. iii). More specifically CSeSP and HAISP MSCs demonstrated a significant increase in calcium deposition compared to HA and CS LA populations. There were no significant changes over the Plastic control populations (B).

Figure 3.5: Characterisation of the osteogenic differentiation potential of ECM isolated EA and LA ECM-isolated MSCs compared to the ECM-isolated MSC sub-populations: (A) Light microscopy analysis of MSC populations showed positive Alizarin Red staining demonstrating deposition of a calcified osteogenic matrix. The undifferentiated controls (iii) were shown to be negative for matrix staining, Scale bar = 200 μm.
Figure 3.5: Characterisation of the osteogenic differentiation potential of ECM isolated EA and LA ECM-isolated MSCs compared to the ECM-isolated MSC sub-populations: (B)

Quantification of calcium deposition per well by the various cell types demonstrated a significant increase in osteogenic differentiation potential in CSeSP MSCs compared to the HA and CS LA populations (p>0.05). HAISP MSCs also showed increased calcium deposition compared to the HA LA MSCs (p>0.05). * indicate p≤0.05; ** p≤0.005. Data is presented as fold change from plastic-adherent cultures. Values are presented as the mean ± SD of data from 3 donors.
3.3.6 Characterisation of Chondrogenic Differentiation Potential of ECM-isolated Populations and Sub-Populations

Chondrogenic differentiation was carried out to determine if the HA and CS sub-populations were depleted in chondrogenic progenitor cells. In the previous chapter, we demonstrated an approximate 4-fold enhancement in chondrogenesis in the HA EA and CS EA populations (Figure 2.8. A). Here, the chondrogenic ability of eSP MSCs was determined to assess if this enrichment resulted in a reduction of chondrogenic potential in the side population.

Differentiation was assessed in all populations of MSCs after they were pelleted and treated with CCM for 21 days of culture (Figure 3.6 A). The chondrogenic pellets were histologically analysed and positive results demonstrated an increase in pellet size and staining due to the increased production of GAG (A. 1,2,3. i-ii, iv-v). GAG was visualised by staining with Safranin O. Despite all treated groups staining positively for proteoglycans, the HAeSP pellet stained more intensively for Safranin-O and was a larger pellet in terms of volume compared to the other MSC groups (A. 2. ii).

The HAeSP pellets displayed more proteoglycan throughout, with more positive stain towards the outside of the pellet than the other treatment groups as shown in the enlarged images in Figure 3.6 B. Due to the excess production of GAG, the pellet is visibly larger than the other treatment groups (B. 2. ii) The pellets size and their staining correlate with the results shown in the GAG quantification graphs. The negative group demonstrated malformed pellets, completely devoid of Safranin-O staining (A. 1,2,3 iii).

Chondrogenesis was quantified for GAG per DNA content and a significant increase was observed in HAeSP populations compared to all other populations and sub-populations except CSeSP, CS LA and CSISP. There was an approximate 9-fold increase in the HAeSP MSCs over the plastic isolated MSCs (P EA, PeSP, P LA and PISP). There was no significance in the CS populations due to the large variations between the human donors used.
Figure 3.6: Characterisation of the Chondrogenic Differentiation Potential of ECM-Isolated MSC populations Compared to MSC Sub-populations: (A) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans produced by chondrocytes. Sulfated GAG was present in all ECM isolated MSC populations. The undifferentiated controls that received incomplete chondrogenic medium without TGF-β3 (A. 1,2,3 iii) were shown to be negative for both the Safranin O stain and the rounded phenotype of positive chondrogenic pellets. Scale bars = 500 μm.
Figure 3.6: Characterisation of the Chondrogenic Differentiation Potential of ECM-Isolated MSC populations Compared to MSC Sub-populations: (B) Expanded images of the early adherent and early sub-population pellets to illustrate the large quantity of GAG produced in the HAeSP pellets compared to the PeSP and also compared to the HA EA pellets. This HA EA MSC population was previously shown to have significantly higher chondrogenic differentiation potential than control MSCs (figure 2.9). Scale bars = 500 μm.
Figure 3.6: Characterisation of the Chondrogenic Differentiation Potential of ECM-Isolated MSC populations Compared to MSC Sub-populations: (C.) Glycosaminoglycan per DNA content was quantified for all ECM isolated MSC populations and sub-populations. Chondrogenesis was significantly higher in HAeSP MSCs with an approximate 9-fold increase over all other plastic controls (p≤0.001) and a significant increase over the other MSC populations except CS LA and CSISP. * indicate p≤0.01; ** p≤0.001. Data is presented as fold change from plastic-adherent cultures. Values are presented as the mean ± SD of data from 4 donors.
3.3.7 Differential Expression of CD44 Receptor between Populations

CD44 is the main receptor for both HA and CS expressed by cells (Aruffo et al., 1990). The expression levels of the MSC surface receptor CD44 were analysed by flow cytometry to assess any changes in the expression levels of the receptor after ECM molecule exposure and subsequent culture. There were no differences shown in the EA or LA populations as shown in the previous chapter (Figure 2.10), nevertheless it was important to analyse whether the sub-populations demonstrated differences in CD44 receptor expression. There were however, no significant changes in CD44 expression levels on the cell surface of MSCs isolated using adherence to HA and CS or the sub-populations exposed to HA and CS (Figure 3.7) compared to traditionally isolated methods.

Figure 3.7: Cell Surface Expression Levels of CD44 on ECM Isolated and Sub-population MSCs: There was no statistical difference in the cell surface expression levels of CD44 between ECM isolated MSCs or sub-populations (p>0.5). Data is presented as fold change from plastic-adherent cultures. Values are presented as the mean ± SD of data from 3 donors.
3.3.8 CFU-F Analysis of EA Populations and eSP Populations

As described in Figure 2.4, one of the criteria to define an MSC includes the ability of the heterogeneous, adherent population in the marrow to form colonies. Each colony is considered to derive from a single cell (Horwitz, Le, Dominici et al., 2005) and colonies are counted once 10-15 cells are present. This assay was carried out to analyse the colony forming abilities and characteristics of the early sub-populations and to determine if they were altered compared to the EA populations. Only HA populations were included in this analysis as CSeSP cells were not found to have increased chondrogenic potential compared to controls. On the other hand HAeSP MSCs displayed the highest chondrogenic potential when compared to all other MSC populations (Figure 3.6).

CFU-F analysis indicated that there was no significant difference in the ability of PEA, PeSP, HA EA and HAeSP MSCs to form colonies. Significant variation between donors resulted in large error bars within the groups. However, the lack of statistical significance reveals that there remains a similar number of CFU-Fs available in the BM wash-off and that the potent chondroprogenitors present in this fraction are fully capable of adhering and forming colonies, albeit slightly later than the initial fraction. Macroscopic images were taken of the flasks containing the CFU-Fs stained with Crystal Violet to enable counting of the colonies (Figure 3.8). Colonies can be clearly seen with defined edges. The eSP cultures show a trend to a lower number of colonies forming in the dishes. Despite this, these sub-population colonies have proven to be fully proliferative. As shown in Figure 3.3, these eSP culture’s grow at about the same rate as the early adherent cultures.
Figure 3.8: Colony forming unit-fibroblasts: Graphical presentation of the colony forming units of PE A, PeSP, HA EA and HAeSP MSCs. The CFU-F number did not vary significantly between isolation methods. The images show corresponding macroscopic images of crystal violet stained CFU-Fs. P>0.1; Values are presented as the mean ± SD of data from 5 donors.
3.3.9 Analysis of SOX9 Expression in PeSP and HAeSP
Chondrogenically Induced Pellets

As SOX9 was shown to be increased in HA EA cells during chondrogenesis in Figure 2.12, it was necessary to analyse SOX9 expression in HAeSP cells during chondrogenic differentiation as the chondrogenic ability in this cell population was significantly increased when measured by GAG production. The PeSP and HAeSP MSCs were pelleted in CCM for 3 minutes (Day 0) and for 24 hours (Day 1), to measure the temporal expression of SOX9, the master transcription factor for chondrogenic differentiation of hMSCs (Akiyama et al., 2002). This was carried out using qPCR and analysed using the ΔΔCT Method, where 18S acted as the endogenous control and PeSP T0 acted as the calibrator. The HAeSP cells demonstrated a 20-fold increase in SOX9 expression compared to the plastic sub-population- PeSP (Figure 3.9). The HA EA SOX9 expression shown in Figure 2.14 demonstrated an approximate 3-fold increase over the P EA cells whereas the HAeSP demonstrated a 20-fold increase over the PeSP which had similar expression to the P EA.
Figure 3.9: PCR analysis of SOX9 expression in Day 0 and 1 chondrogenic PeSP and HAeSP pellets: qRT-PCR analysis was carried out on the HAeSP and the PeSP control populations to analyse SOX9 transcription factor expression immediately after pelleting at day 0 and after 24 hours in chondrogenic medium (day 1). HAeSP chondrogenic pellets were shown to have significantly higher levels of SOX9 compared to all other cell populations, *** indicates p≤0.0001. Values are presented as the mean ± SD from 3 donors.
3.3.10 Analysis of SOX9 Expression in HAeSP Monolayer Cells compared to PeSP

It was necessary to analyse SOX9 expression in monolayer cells (cells before chondrogenic pellet induction) because it appears that the HAeSP cells are activated or selected by the presence of HA; thus, SOX9 may have been expressed during expansion culture prior to chondrogenic induction in pellets. qRT-PCR analysis was carried out and analysed using the ΔCT Method, where Drosha acted as the endogenous control. This analysis did not demonstrate an up-regulation in SOX9 expression in HAeSP monolayer cells; moreover there was a low, basal level expression of SOX9 in both MSC populations (Figure 3.10).

Figure 3.10: PCR analysis of SOX9 expression in monolayer EA and eSP MSCs: PCR analysis was carried out on the P EA, PeSP and the HA EA and HAeSP undifferentiated MSCs to determine if SOX9 is expressed in monolayer cells without chondrogenic induction or the condensation of 3D pellets. There were no differences in SOX9 expression demonstrated between groups (p>0.05). Values are presented as the mean ± SD of data from 3 donors.
3.4 Discussion

Human MSCs demonstrate regenerative capacity and multipotentiality and therefore have gained importance in tissue engineering and other clinical therapies such as myocardial infarction, osteogenesis imperfecta, graft versus host disease (GVHD), spinal cord injury and diabetes (Wollert et al., 2011; Otsuru et al., 2012; Pérez-Simon et al., 2011; Mothe et al., 2012; Gabr et al., 2011; http://www.clinicaltrials.gov/). *Ex vivo* amplification prior to these clinical applications to obtain therapeutic doses is essential due to the limited availability of MSCs in the BM (0.001-0.01% total nucleated cells) (Caplan et al., 1994). As defined in an ISCT position paper (Dominici et al., 2006), MSCs must be plastic adherent when maintained *in vitro*, must be capable of tri-lineage differentiation and ≥95% of the population must express CD73, CD90 and CD105. Generally, clinical practices employ cell culture protocols and technologies where a small fraction of primary hMSCs are isolated from a selected tissue source and expanded over multiple passages to generate a sufficient number of cells for clinical use. This leads to high production costs and the safety and efficacy of cell therapeutics produced may be negatively influenced by cell bioprocessing protocols such as quality control of *in vitro* cultured MSCs and obtaining enough cells while maintaining appropriate cell passages (Horwitz et al., 2011; Wu et al., 2012).

Consequently, it is critical to develop robust production processes by optimizing culture protocols to efficiently, quickly and consistently generate human MSCs which retain their desired regenerative and differentiation properties, while at the same time minimise potential risks to patients. Translation of pre-clinical studies, quite often using poorly defined MSC populations, to use in patients will require clinical-grade, large-scale MSC expansion with precise definition and standardisation of the procedural parameters (Fekete et al., 2012).

In this study, a novel time-frame and process to enhance chondroprogenitor isolation from whole bone marrow has been developed. The collection of non-
adherent sub-populations allowed the yield of BM-derived MSCs to be increased as the number of cultures which were derived doubled (Figure 3.3). The method allows a significantly higher number of MSCs to be isolated in primary culture. The method employed here seemed to have a better outcome than previous studies where a yield increase of 36-37% was found using the non-adherent cell population (Wlodarski et al., 2004; Wan et al., 2006; Leonardi et al., 2009). Furthermore, the newly developed method allowed the isolation of a more potent chondroprogenitor population.

Through phenotypic and functional analysis we have demonstrated that the plastic, HA and CS population’s and sub-population’s characteristics are very similar. It was shown in a study by Leonardi et al. that in primary culture, over 60% of cells replated onto plastic after 4 days in culture were positive for CD45 pan-leukocyte antigen which disappears after subsequent passages (Leondardi et al., 2009). Based on this study, a possible explanation for the delay in the adherence process of our sub-populations could be due to the high proportion of cells belonging to the hematopoietic lineage found in the sub-populations, therefore this could affect adherence when these cell are all competing for the same surface area in a single tissue culture flask.

After one day culture of BMMNCs, the presence of non-adherent MSCs for establishment of early sub-population cultures is not unexpected. However, it was not expected that there would be functional MSCs remaining non-adhered in the marrow cultures plated for 5 days. These late sub-population cells (along with all other populations) were shown to be fully functional in terms of colony forming ability, proliferation, MSC surface expression and differentiation (Figures 3.2-3.9 and Table 2). Molchanova et al have recently shown that, although MSCs from BM adhere within the first 7 days to establish cultures, approximately 60% of CFU-Fs remain in suspension and their clonogenic ability is realized during sequential transfer into a new tissue culture dish. It was shown that the total number of CFU-Fs from BM was much greater than that calculated from a seven day attachment period (Molchanova et al., 2011). We have built on this study to analyse the non-
adherent populations that have been exposed to the ECM environment upon initial plating directly from bone marrow.

Additionally, in this study it was important to analyse the sub-population’s ability for tri-lineage differentiation. Although ECM-isolated adherent populations had significantly increased chondrogenic activity as described in Chapter 2, it was not known whether the selection process resulted in a higher ratio of CFU-Fs that are chondroprogenitors attaching in the one day time frame or whether exposure to HA and CS activated the adherent cells. Nevertheless, it was initially hypothesised that there would be a reduction in the amount of chondroprogenitor cells in the sub-populations of the HA and CS isolated MSCs. The approximate 9-fold increase in chondrogenic potential of MSC cultures established from the HA-exposed, early non-adherent mononuclear cells was therefore unforeseen (Figure 3.6). It appears that the cells do not require initial adherence to the HA immobilized on the tissue culture flask to be selected or activated from the marrow. HA is very soluble and it is not unexpected that a portion of the HA bound to the flask would become solubilised in the marrow once it was plated. This would enable attachment or interaction of the non-adhered cells with HA.

The adipogenic ability of the sub-populations was not significantly altered as compared to the EA and LA populations. However, osteogenic differentiation was shown to be significantly increased in the CSeSP and the HAlSP populations but only as compared to the LA ECM isolated populations. This, again, is not entirely surprising as it has been shown in others studies that late adhering and non-adherent populations are enriched for osteoprogenitors (Wlordaski et al., 2004; Leonardi et al., 2009).

It is interesting that there were no significant differences in CFU-F between early populations and sub-populations (Figure 3.8). Although average CFU-F number was lower in the latter populations, the data reiterates the phenomenon that there are many functional non-adherent cells still present in the bone marrow after 24 hours and 5 days. Wan et al also demonstrated MSC cultures derived from the non-
adherent cell population of human bone marrow had very similar proliferation and differentiation rates to the primary adherent populations (Wan et al., 2005). This is an important observation as it highlights technologies that may result in significantly increased yield and potency of bone marrow MSC preparations.

Molecular analysis showed that expression of SOX9 was approximately 20-fold higher in the HAeSP MSCs compared to the PeSP MSCs after a 24 hour exposure to chondrogenic medium. This was a highly significant increase over the plastic sub-population (Figure 3.9). Taken together, the significant increases that were demonstrated for proteoglycan deposition after 21 days and early SOX9 expression reveal that a highly chondrogenic population is isolated from the HA-exposed early sub-population. This population is also significantly more chondrogenically active than the HA EA population isolated in the chapter 2. This could lead to a new method of chondroprogenitor isolation with higher numbers of MSCs isolated at the same time.

SOX9 expression was also tested in monolayer cells to analyse whether it is expressed in cells prior to chondrogenic induction in a 3D format (Figure 3.10). There was no increase in SOX9 between cell populations prior to induction of chondrogenesis. This data may indicate that the chondrogenic MSC populations cells are primed by exposure to HA to respond to the signalling events involved in initiation of chondrogenesis.

It also became apparent that the initially non-adherent cells acquire the ability to adhere under altered conditions i.e., depletion of the competing MSCs obtained in vitro after their early adhesion. Hence, they become adherent after secondary plating when competition for binding is lower.

Despite the high chondrogenic potential of the HAeSPs, the PeSP MSCs exhibit chondrogenic properties very similar to the P EA MSCs as well as similar proliferative capacity. Thus, beyond isolating an extremely effective chondrogenic cell, this study also points out the benefits of enhancing the numbers of MSCs by
retrieving and re-plating the non-adherent cell population of bone marrow MSC culture. This enhancement in numbers is carried out without functional differences or disadvantages occurring to the cells compared to the typical MSCs isolated in laboratories for varying research purposes.

We demonstrated in chapter 2 that HA does not exert the same chondro-enhancing effects on cells that are already passaged (Figure 2.11. B), therefore, it is important to analyse the effects of HA in marrow and why the HA requires the presence of the bone marrow in order to exert its effects on the cells.
Chapter 4

Analysis of Different Methods of ECM Exposure to Bone Marrow

Identification of a Synergistic Relationship between Cartilage ECM Molecules and the Bone Marrow Niche upon Chondroprogenitor Isolation.
4.1 Introduction

The stem cell niche is an anatomical site that contains a reservoir of stem cells that can maintain healthy tissue or replenish aged cells in response to mechanisms that regulate the cell responses such as quiescence, self-renewal or differentiation (Vazin and Schaffer, 2010). Therefore, MSCs can be influenced or activated by signals from the local micro-environment. Menon et al have looked at specific micro-environmental effects on MSC function and gene expression (Menon et al., 2007). They described how a subset of 12 genes were down-regulated when rat MSCs were exposed to bone marrow conditioned medium. These genes included CXCL-12, SDF-1, CXCL-2, CINC-2, endothelial cell specific molecule-1, fibroblast growth factor-7, nuclear factor-B p105, and thrombomodulin. In contrast they also demonstrated that this same subset of transcripts were found to be upregulated in rat MSCs that were exposed to tumour cell conditioned medium. This demonstrated that MSCs undergo specific alterations in gene expression and regulation patterns in response to different microenvironments and that these changes influence important MSC functions such as differentiation.

From these studies, it can perhaps be hypothesised that the molecular mechanisms underlying the activation of MSCs can be mediated by signals from the marrow environment, and that the specific cytokines or paracrine factors in the HA micro-environment interact with the MSC surface and activates an MSC fate decision. This could then lead to the increased chondrogenic differentiation capacity in the HAeSP MSC population. It was therefore hypothesised that;

1. Adding HA directly to the whole BM increases the chondrogenic potential of MSCs.

2. There is a relationship between HA and the BM environment that enhances the effect of HA on MSCs in whole BM, thus enhancing chondrogenic potential.
The specific micro-environments of stem cell niches regulate stem cell function by providing architectural support, along with humeral and cell-contact dependent signals (Vazin and Schaffer, 2010; Scadden et al., 2006). Recent studies have analysed the stem cell niche and have revealed that cell types such as endothelial cells, osteoclasts and mesenchymal progenitors are imperative in establishing a niche’s function. It appears from these studies that for the emulation of active niches in vitro, there may be a need for more than concoctions of various cytokines. Perhaps, the reconstruction of an environment which includes cellular players and deposition of a true physiological extracellular matrix would result in cells acting as required. As outlined in the Lander et al review, niches may be composed of cells, or cells along with the extracellular matrix structures. The niche may be a source of cell surface or secreted factors such as members of Wnt, FGF, TGF-β, Notch, epidermal growth factor (EGF), stem cell factor (SCF) and the chemokine families. These factors work to control stem cell maintenance, differentiation and/or survival (Lander et al., 2012; Morrison et al., 2008; Lam et al., 2006). The niche may consist of one cell type or a host of different cells and it may be derived from the stem cell family or from cells outside the stem cell lineage. A consensus among niche studies is that stem cells specifically require the niche for their maintenance (Morrison et al., 2008; Oshima et al., 2001; Wilson et al., 2006). The reason that stem cells require this support and other cells do not may be due to the demands that are placed on stem cells, for example, the need to minimise accumulation of genetic damage due to their pluripotent states. This pressure on the cell’s metabolic activity may necessitate special support and sustenance from their micro-environment. There is also the possibility that the cells require feedback control because stem cell pools are usually capable of expanding and contracting and can sometimes face large stochastic fluctuations under certain homeostatic conditions. A third possibility outlined by Lander et al is that the niche may be an instrument of coordination among tissue compartments because there is a need for strict control over the different cell populations within a particular organ in order to achieve proper coordination amongst the cells (Lander et al., 2012).
From results shown in Chapter 2, it was considered that HA exposure in culture does not affect the chondrogenic potential of passaged MSCs but only when HA is present during the isolation of MSCs from the bone marrow. Based on this rationale, we assessed whether there is a relationship between HA and the marrow niche that enhances HA’s effect on MSCs in the marrow, thus contributing to the enhancement in chondrogenic potential. MSCs were isolated as described in Chapter 3, but after a 24 hour period in culture, the HA conditioned BM was removed and added to the cells that were not conditioned with HA within the first 24 hours in culture. Cells were subsequently analysed for chondrogenic potential.

Furthermore, as demonstrated in Chapter 3, cells do not in fact need to adhere to the immobilized HA as the non-adherent populations and the subsequent sub-populations isolated had an enhanced chondrogenic phenotype. We therefore assessed whether the addition of HA directly to the whole BM also enhanced the chondrogenic potential of isolated MSCs.
4.2 Materials and Methods

All materials were supplied by Sigma Aldrich unless otherwise stated.

4.2.1 Exposure of Unprocessed Bone Marrow to HA for 24 hours

As described in Section 2.2.1; human mesenchymal stem cells were isolated from the bone marrow of the iliac crest of healthy donors and all procedures were performed with informed consent and ethically approved by the Clinical Research Ethical Committee at University College Hospital, Galway.

Before processing, HA was added to the BM. The BM was first split into two equal volumes and each part was transferred into fresh 50 ml tubes. 1mg/ml of Durolane HA was found to have the best effect in solution from previous studies carried out (chapter 2). Thus, 1 mg/ml HA was added to serum free α-MEM and this was then added into one tube of BM. The other tube of BM received serum-free α-MEM only. These were secured with parafilm and left at room temperature in the dark overnight. After 24 hours the BM in the tubes was centrifuged at 900 x g for 10 minutes to ensure as many cells as possible were retained in the pellet and to remove as much of the HA as possible.

Following normal BM processing as outlined in Section 2.2.1, the cells were plated on T175s with complete expansion medium plus 5 ng/ml FGF-2 (R&D Systems). The HA in the marrow was not fully removed when centrifuged as the solid particles in the HA were mixed with the cells (Figure 4.1).
Figure 4.1: Before processing, the BM was split into two equal volumes. 1mg/ml of HA was added to serum free α-MEM and this was then added into one tube of BM. The other tube of BM received serum-free α-MEM only. These were secured and left at room temperature, in the dark overnight. After 24 hours at room temperature and in the dark, the marrow samples were processed as described (Section 2.2.1) and MNC plated and expanded to P1 for determination of SOX9 gene expression and tri-lineage differentiation analysis.
4.2.2 Co-activator Activity in Marrow

HA exposure in culture does not affect the chondrogenic potential of passaged MSCs. When HA is present upon the isolation of MSCs from the marrow, there is a significant increase in chondrogenic potential. Our resulting hypothesis is that there is a synergistic relationship between HA and the marrow that enhances the effect of HA upon MSCs in the BM, thus enhancing chondrogenic potential.

After processing the marrow as described above, the marrow was plated as usual on a HA coated flask (the non-coated flask acting as the control throughout). After 24 hours of culture, the marrow and non-adhered cells were removed from the dishes and transferred into a 50 ml tube. The dishes were washed with non-serum containing α-MEM and the washes were added into the same tube. A quarter of the marrow wash was taken from this tube and plated back onto a non-coated dish to isolate the early sub-population (HAeSP). The other three quarters were centrifuged at 900 x g for 10 minutes to form a separate HAeSP pellet and HAeSP cell supernatant. The supernatant was then removed and placed into a new tube. The pellet was resuspended in 15 mls of complete MSC medium. One third of this suspended pellet was plated onto a non-coated dish and supplemented with supernatant from the HAeSP cell conditioned marrow. The remaining two thirds of the HAeSP suspension were plated into equal parts on two non-coated dishes. One of these was supplemented with plastic control (PeSP) marrow supernatant and the other was supplemented with expansion medium only and was not exposed to marrow again. The exact opposite was carried out on the PeSP MSCs, i.e. the PeSP cells were supplemented with HAeSP cell marrow supernatant etc. (Figure 4.2).
Populations arising from this experiment are:

- HA EA cells
- HAeSP cells
- HAeSP cells with the HAeSP cell supernatant added back in after spin (HAeSP+HAeSP Supernatant)
- HAeSP cells with the PeSP cell supernatant added (HAeSP+PeSP Supernatant)
- HAeSP cells without any supernatant added (HAeSP+non-conditioned Medium Only)

Control Populations:

- P EA cells
- PeSP cells
- PeSP cells with the PeSP cell supernatant added back in after spin (PeSP+PeSP Supernatant)
- PeSP cells with the HAeSP cell supernatant added (PeSP+HAeSP Supernatant)
- PeSP cells without any supernatant added (PeSP+Medium Only)
**Figure 4.2:** After processing, the marrow was plated as usual on a HA coated flask (the non-coated flask as the control). After 24 hours in culture, the bone marrow and non-adhered cells were removed from the dishes and transferred into a 50ml tube. A quarter of the marrow wash was plated back onto a non-coated dish to create the early sub-population (HAeSP). The other three quarters were centrifuged to form a separate HAeSP pellet and HA-marrow supernatant. The supernatant was placed into a new tube. The pellet was resuspended in complete MSC medium. One third of this suspended pellet was plated onto a non-coated dish and supplemented with supernatant from the HAeSP marrow. One of the remaining two thirds was supplemented with plastic control marrow (PeSP) supernatant and the other was supplemented with expansion medium only and was not exposed to marrow again. The exact opposite was carried out on the PeSP MSCs, i.e. the PeSP cells were supplemented with HAeSP marrow supernatant etc.
4.2.3 Populations Obtained from Process to Elucidate Synergistic Relationship between Marrow and HA

Outlined in the table below are the populations which arise from the previous experiment (4.2.2) carried out to analyse if there is a synergistic relationship between bone marrow and HA to chondrogenically activate the MSCs. All populations except for the HA EA and P EA are derived from the HAeSP MSCs or PeSP MSCs; they differ by the marrow conditioned media with which they are treated with.

Table 4.1: Outlines the populations which arise from the co-activator activity experiment (PL- abbreviation for plastic –non coated control).
Analysis of Cell Populations

Analysis of cell populations was carried out as described in materials and methods chapter 2, sections 2.2.5.1-2.2.5.3 describing adipogenic, osteogenic and chondrogenic differentiation of cell populations. PCR was carried out as described in section 2.2.10

4.2.4 Statistical Analysis

All values are presented as the mean ± standard deviation of the mean (SD). Data sets were tested for significance using the One-Way ANOVA (non-parametric) and Tukey post-test to compare between groups. A level of p ≤ 0.01 or 0.05 was considered statistically significant. These statistical analyses were used unless otherwise stated.
Chapter 4 Part 1:

Analysis of Direct HA Exposure to Bone Marrow
4.3 Results

4.3.1 SOX9 Expression in Chondrogenic Pellets; HA-Conditioned BM MSCs Compared to the Non-Conditioned BM MSCs

SOX9 expression was measured at the end of passage 1 in HA-conditioned or non-conditioned cells in order to analyse if the cells were activated in a similar fashion to previous results (Section 2.3.8 and 3.3.6) but here the HA was dissolved in the marrow before processing and not coated on tissue culture flasks. SOX9 is the main transcription factor associated with chondrogenesis which peaks at approximately 24 hours after chondrogenic induction (Akiyama, H et al., 2002). The cells were pelleted in CCM for 3 minutes (Day 0-D0) and for 24 hours (Day 1-D1), to measure the temporal expression of SOX9. qRT-PCR demonstrated a significant increase in the HA-conditioned day 1 chondrogenic MSCs as compared to the non-conditioned MSCs (Figure 4.3).
**Figure 4.3: PCR analysis of SOX9 expression in HA-Conditioned and Non-Conditioned BM:**
PCR analysis was carried out on the 24 hour HA-Conditioned population and the Non-Conditioned control population to analyse SOX9 transcription expression at 0 hours (D0) and 24 hours (D1) in chondrogenic differentiation conditions. The HA-Conditioned D1 chondrogenic pellet was shown to have significantly higher levels of SOX9 compared to the Non-Conditioned chondrogenic pellet at D1. ** indicates p≤0.001; *** indicates p≤0.0001. Values are presented as the mean ± SD of data from 3 donors.
4.3.2 Chondrogenic Differentiation in HA-Conditioned BM MSCs Compared to the Non-Conditioned BM MSCs; GAG deposition

The outcome at a later time-point was analysed in order to determine if the MSCs remained as highly chondrogenically active throughout the three week chondrogenic process. Figure 4.4 demonstrates significantly higher chondrogenic ability in conditioned MSCs compared to the non-conditioned MSCs, measured by the GAG / DNA ratio (Figure 4.4. B). This result correlates with the SOX9 expression data shown in figure 4.3 at day 1 in chondrogenic differentiation. The chondrogenic pellets were histologically analysed and results demonstrated an increase in pellet size and staining due to the increased production of GAG in the HA-Conditioned population compared to the pellet from the Non-Conditioned population (A). GAG was visualised by staining with Safranin O. The blue colour shows a negative result for the presence of proteoglycans.

![Figure 4.4: Analysis of the chondrogenic differentiation potential of MSC populations from HA-Conditioned BM compared to populations from Non-Conditioned BM: (A) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans produced by chondrocytes. Sulfated GAG was present in both populations, with more GAG staining observed in the HA-conditioned MSC population. Scale bar = 500 μm.](image)
Figure 4.4: Analysis of the chondrogenic differentiation potential of MSC populations from HA-Conditioned BM compared to populations from Non-Conditioned BM: (B) Glycosaminoglycan per DNA content was quantified for HA-Conditioned BM MSC populations. There was a significant increase in chondrogenic differentiation capacity of the MSCs conditioned with HA in the marrow compared to the non-conditioned population. * indicate $p \leq 0.01$ using Student’s t-Test; Data is presented as fold change over non-conditioned control pellets. Values are presented as the mean ± SD of data from 3 donors.
4.3.3 Adipogenic Differentiation in Non-Conditioned Compared to the HA-Conditioned MSCs

Previous experiments have shown that the presence of HA does not alter the adipogenic differentiation of MSCs. It was therefore necessary to analyse if the conditioning of whole BM with HA also had no effect on the adipogenic differentiation of isolated MSCs. Adipogenesis was quantified through Oil Red O extraction from the adipocytes. Oil Red O staining of intracellular lipid vacuoles identified adipocytes in induced adipogenic cultures of the MSC populations Figure 4.5 A). Lipid quantification demonstrated that there was no significant difference in adipogenesis between HA-conditioned and non-conditioned MSC populations (B).

Figure 4.5: Characterisation of the adipogenic differentiation potential of MSCs from 24 hour HA-Conditioned BM compared to populations from the Non-Conditioned BM: (A)
Light microscopy analysis of both populations was positive albeit low for Oil Red O staining of the lipid vesicles. Scale bar = 200 μm.
Figure 4.5: Characterisation of the adipogenic differentiation potential of MSCs from HA-Conditioned BM compared to populations from the Non-Conditioned BM: (B)

Quantification of Oil Red O extraction from both populations indicated no significant difference in adipogenic potential between the populations (p>0.1) using Student’s t-Test. Values are presented as the mean ± SD of data from 3 donors.
4.3.4 Osteogenic Differentiation in Non-Conditioned Compared to HA-Conditioned MSCs

Osteogenic differentiation was analysed by an assay for calcium deposition. Alizarin Red staining revealed slightly enhanced matrix deposition in the non-conditioned MSC population (Figure 4.6. A). Calcium deposition was shown to be significantly enhanced in the non-conditioned population demonstrating that osteogenic differentiation is significantly reduced in the HA-conditioned population (Figure 4.6 B).

*Figure 4.6: Characterisation of the osteogenic differentiation potential of MSCs from HA-Conditioned BM compared to populations from the Non-Conditioned BM: (A) Light microscopy analysis of MSC populations showed positive staining by Alizarin Red of osteocyte matrix deposits in the non-conditioned MSC population. The HA-conditioned MSCs were shown to be low for matrix staining. Scale bar = 500 μm.*
Figure 4.6: Characterisation of the osteogenic differentiation potential of MSCs from HA-Conditioned BM compared to populations from the Non-Conditioned BM: (B) Quantification of calcium deposition by the cells per well demonstrated a significant increase in osteogenic differentiation potential in the non-conditioned population. * indicate p≤0.01 using Student’s t-Test; Values are presented as the mean ± SD of data from 3 donors.
Chapter 4 Part 2:

Synergistic Effect of BM and HA on Chondroprogenitor Activation
4.3.5 Adipogenic Differentiation in the Marrow Conditioned Media Treated Populations

It was necessary to analyse if there were any differences in tri-lineage differentiation potential upon treatment with the conditioned media. Oil Red O staining of intracellular lipid vacuoles identified adipocytes in induced adipogenic cultures of the MSC populations (Figure 4.7 A). Adipogenic differentiation followed the trend of little difference in differentiation capacity between treated populations; there were no significant differences in differentiation potential of the populations described in this experiment (B).

Figure 4.7: Characterisation of the adipogenic differentiation potential of PeSP, HAeSP and conditioned media treated populations: (A) Light microscopy analysis of MSC populations was positive, albeit low for Oil Red O staining of lipid vesicles while the undifferentiated control MSCs were negative for Oil Red O staining. *Scale bar = 200 μm.*
Figure 4.7: Characterisation of the adipogenic differentiation potential of PeSP, HAeSP and conditioned media treated populations: (B) Quantification of Oil Red O extraction from all populations indicated no significant difference in adipogenic potential between PeSP, HAeSP or any of the conditioned medium treated populations and sub-populations (p>0.1). Values are presented as the mean ± SD of data from 3 donors.
4.3.6 Osteogenic Differentiation in Marrow Conditioned Media Treated MSCs

Osteogenic differentiation was analysed by assessing calcium deposition. Alizarin Red staining revealed enhanced matrix deposition in the “HAeSP+Medium Only” and “PeSP+Medium Only” populations (Figure 4.8 A). Corresponding to this semi quantitative analysis, calcium deposition was shown to be low in all treatment groups apart from the non-marrow conditioned treatment groups; “PeSP+Medium Only” and “HAeSP+Medium Only” populations. These two treatments showed significantly higher osteogenic differentiation potential (Figure 4.8 B).

![Matrix Staining of Osteogenic Differentiated Cells](image)

**Figure 4.8: Characterisation of the osteogenic differentiation potential of PeSP, HAeSP and conditioned media treated populations:** (A) Light microscopy analysis of MSC populations showed positive staining by Alizarin Red of osteocyte matrix deposits; there were more matrix deposits in the medium only populations. The undifferentiated control was shown to be negative for matrix staining. Scale bar = 500 μm.
Figure 4.8: Characterisation of the osteogenic differentiation potential of PeSP, HAeSP and conditioned media treated populations: (B) Quantification of calcium deposition demonstrated a significant increase in osteogenic differentiation potential in non-marrow conditioned medium treated MSCs, both “HAeSP+Medium Only” and “PeSP+Medium Only” (p>0.0001). *** ps0.0001; Values are presented as the mean ± SD of data from 3 donors.
4.3.7 SOX9 Expression Analysis in Marrow Conditioned Media Treated MSCs

SOX9 expression was analysed as an early event of chondrogenic alteration upon treatment with “HAeSP or PeSP marrow conditioned media”, where isolated PeSP and HAeSP MSCs were resuspended in and cultured with HAeSP conditioned marrow and PeSP conditioned marrow respectively (Figure 4.9). The significant differences obtained from this study were the prevailing increase with the HA isolated populations where the HAeSP sub-populations have consistently shown significantly enhanced SOX9 expression over PeSP populations. Expression of SOX9 in the “HAeSP+PeSP Supernatant” MSC pellets was moderately lower, however, this was not significant. A significant decrease was demonstrated in the “HAeSP+Medium Only” populations.
**Figure 4.9: PCR analysis of SOX9 expression in PeSP, HAeSP and conditioned medium treated populations:** PCR analysis was carried out on all populations to analyse SOX9 transcription expression after 24 hours in chondrogenic differentiation conditions. There were no significant increases in the levels of SOX9 when PeSP or HAeSP cells were treated with marrow conditioned medium (p>0.1). The significant changes were those between the HAeSP and PeSP populations, HAeSP was significantly higher than in PeSP (p < 0.01) and there was significantly lower expression in “HAeSP+Medium Only” (p < 0.01); Values are presented as the mean ± SD of data from 3 donors.
4.3.8 Chondrogenic Differentiation in Marrow Conditioned Media Treated MSCs; GAG Deposition

Differentiation was assessed in all populations of MSCs after they were pelleted and treated with CCM for 21 days of culture (Figure 4.10 A, B).

The chondrogenic pellets were histologically analysed (Figure 4.10. A). GAG was visualised by staining with Safranin O. Despite treated groups (A 1, 2. i, ii and iii) staining positively for proteoglycans, the “HAeSP+PeSP Supernatant” pellet stained less intensively for Safranin-O and was a more malformed pellet than the other HA groups (A 2. iii). Correspondingly the “PeSP+HAeSP Supernatant” pellet stained more intensively than the other PeSP pellets. This “PeSP+HAeSP Supernatant” pellet displayed more GAG throughout, with a stronger stain towards the outside of the pellet than the other PeSP treatment groups (A 1. iii).

Undifferentiated controls (Figure 4.10 A 1, 2. v.) were negative for proteoglycan and had a very small and non-rounded phenotype. Additionally both PeSP and HAeSP cells that were cultured in the absence of marrow after 24 hours plating showed minimal chondrogenesis (PeSP+Medium Only and HAeSP+Medium Only) both resulted in small and malformed pellets, which were negative for proteoglycan (Figure 4.10 A 1, 2. iv).

Chondrogenesis was quantified for GAG/DNA content and once again a significant increase was observed in HAeSP populations compared to the PeSP populations (Figure 4.10 B). This correlated with results shown in Figure 3.6 where the HAeSP population was significantly more chondrogenic than the PeSP population. In agreement with data for the expression of SOX9 after a 24 hour exposure to CCM, HAeSP cells in marrow conditioned medium (HAeSP+HAeSP Supernatant) resulted in a strong and equivalent chondrogenic differentiation as that seen in HAeSP.
Most interestingly, exposure of PeSP cells to the HAeSP marrow conditioned medium (PeSP+HAeSP Supernatant) increased chondrogenesis significantly and conversely, exposing the HAeSP cells to Plastic early Sub-Population (PeSP) marrow conditioned medium significantly decreased chondrogenesis (Figure 4.10 B).

Another noteworthy result was that the “PeSP+Medium Only” and “HAeSP+Medium Only” pellets had low chondrogenic ability and were both significantly lower than their related PeSP and HAeSP treatments, respectively. Taken together, these results show that the marrow environment at initial plating of the MSC is essential for successful chondrogenic differentiation. Also, when traditionally isolated MSCs are treated with marrow containing solubilized HA, it enhances the chondrogenic capacity, and cells isolated on HA have their chondrogenic capacity reduced by removal from the HA environment and treatment with marrow without solubilized HA.
Figure 4.10: Characterisation of the chondrogenic differentiation potential of PeSP, HAeSP and conditioned medium treated populations: (A) Micrographs of chondrogenic pellets stained with Safranin O to visualise sulphated proteoglycans produced by chondrocytes. Sulfated GAG was present in all MSC populations treated with CCM. Medium only exposed MSCs had low levels of stained GAG. The undifferentiated controls were shown to be negative for both the Safranin O stain and the large rounded phenotype of positive chondrogenic pellets. Scale bars = 500 μm.
Figure 4.10: Characterisation of the chondrogenic differentiation potential of PeSP, HAeSP and conditioned medium treated populations: (B) Glycosaminoglycan per DNA content was quantified for all MSC populations. There was a significant increase in the chondrogenic differentiation of PeSP MSCs when treated with HAeSP marrow conditioned medium (PeSP+HAeSP Supernatant) versus all other PeSP groups; * p≤0.01 over PeSP+PeSP Supernatant, ** p≤0.001 over PeSP, *** p≤0.0001 over PeSP+Medium Only. These cells also showed significantly more GAG/DNA content compared with HAeSP cells treated with PeSP marrow conditioned medium, ** p≤0.001. Similarly GAG/DNA content in HAeSP cells was significantly decreased when treated with PeSP marrow conditioned medium, * p≤0.01. There was also significantly more chondrogenesis than the Medium Only treated groups. Values are presented as the mean ± SD of data from 3 donors.
4.4 Discussion

A number of intrinsic and extrinsic cellular mechanisms mediate self-renewal and differentiation in all stem cells. Elements of the stem cell micro-environment and the stem cells themselves make up an anatomical structure that coordinates the production of functional, mature cells. There are other cells present in the stem cell niches which nurture the stem cells and enable them to maintain tissue homeostasis (Moore and Lemischka, 2006). A dialogue occurs between the stem cells and those present in the niche that fulfil lifelong demands for differentiated cells. Drosophila studies have encouraged the resurgence of the concept of the niche (Boyle et al., 2007). Supplemental stem cells lie dormant in specific locations, waiting to be activated by factors present at particular life cycle stages or if an injury occurs to the body. These factors are beginning to be identified. However, there is little definitive information about which cells produce the regulating factors or which cells are present in the niche to support the stem cells (Morrisen and Spradling, 2008). Many niches and stem cells have been shown to depend on signals whose action may function indirectly to maintain niche integrity. These signals are required, in addition to the major primary signal that acts directly on stem cells to promote their maintenance (Forbes et al., 1996).

Recently, many studies have focused on using natural or synthetic biomaterials to create niches or micro-environments to control the behaviour of stem cells and their differentiation towards chondrogenesis and cartilage formation (Dawson et al., 2008; Varghese et al., 2008). These three-dimensional biomaterials serve as scaffolds which can act as templates for tissue formation through organization, intercellular interactions, mechanical forces, and the addition of bioactive molecules (Dawson et al., 2008; Chung et al., 2008, Chung and Burdick, 2009; Thorpe et al., 2010; Kelly et al., 2010). Many studies have looked at the addition of passaged MSCs into HA-containing scaffolds or hydrogels before implantation into a chondral/osteochondral defect. These studies have shown support of the chondrocyte phenotype and production of ECM molecules (Nettles et al., 2004; Liu
et al., 2006; Chung et al., 2006; Kelly, Prendergast, 2006; Solchaga et al., 1999; Buckley et al., 2009). An issue arising from the use of hydrogels and scaffolds is that their composition can affect the outcome; the hydrogel chemistry or scaffold make-up can greatly influence the cells in a number of ways, whether it is commitment to a particular lineage or alteration of gene expression or initiation of apoptotic signals (Chung et al., 2009). The method of exposure (soluble or bound) is also important to MSC fate (Sharma et al., 2007). The use of HA-based scaffolds and hydrogels have shown positive results, however materials can vary significantly and more long term studies are required (Chung et al., 2009). It is also important to note that the safety and efficacy of cell therapeutics may be negatively influenced by cell bioprocessing protocols in which the MSCs are brought through numerous passages before use on scaffolds. Our studies have expanded upon these niche enrichment and mimetic processes by incorporating HA into the isolation of the MSCs from bone marrow.

Looking at Figure 4.1, the aim is to see if adding HA to marrow for 24 hours has a similar effect as the experiments carried out in chapter 2 and chapter 3. These demonstrated highly increased chondrogenic ability by cell adhering directly to HA immobilized on TC flasks (Figure 2.9) and by re-plating the non-adhered MSCs that were exposed to the immobilized HA (Figure 3.6). However it was unknown whether the solid particles of HA remaining in the culture would hinder the proliferation or differentiation of the cells after direct bone marrow HA exposure because these solid particles are usually removed before marrow plating. Interestingly, the proliferation and adhesion of the 24 hour direct bone marrow conditioned MSCs were normal. Chondrogenic differentiation was significantly increased, affirming that it is not necessary for the cells to attach to immobilized HA coated on a flask to be chondrogenically activated (Figure 4.3 and 4.4). This was shown to be solely a chondrogenic phenomenon as adipogenic and osteogenic differentiation were not positively affected (Figure 4.5 and 4.6 respectively). The osteogenic differentiation of the HA-conditioned MSCs was in fact significantly decreased. Several reports indicate that there are potent osteogenic progenitors present as non-adherent cells in the marrow (DiMaggio et al., 2012, Leonardi et al.,
2009, Wlodarski, Galus and Wlodarski 2004, Muraglia et al., 2000). Thus, it appears that the HA may re-direct these potent progenitors down the chondrogenic lineage upon its exposure to the MSCs and their marrow micro-environment.

The marrow conditioned media experiment was carried out to determine if there is a synergistic effect between the HA and the marrow that is essential for the chondrogenic activation of MSCs following their exposure to HA. It was hypothesized that there was a synergy between them due to HA having no effect on MSCs previously already isolated and passaged (Figure 2.13). It was also thought that the PeSP cells would have a higher chondrogenic potential when treated with HA-marrow conditioned medium and that the HAeSP populations would be chondrogenically inhibited when treated with the PeSP-marrow conditioned medium. This would demonstrate that the bone marrow conditioned with HA can in fact activate plastic isolated MSCs, unlike HA alone, which cannot. This was demonstrated in Figure 4.10 where PeSP cells demonstrate a significant increase in chondrogenesis upon the addition of HAeSP-marrow conditioned medium (PeSP+HAeSP supernatant). The exact opposite was demonstrated when HAeSP MSCs were treated with marrow conditioned medium from the PeSP cells. This demonstrates that bone marrow is a key factor during the induction of chondroprogenitors using HA. Perhaps, it is the abundance of signals and growth factors in the bone marrow along with the presence of HA that directs the cells towards chondrogenesis as it would in true physiological conditions during cartilage formation. It would be interesting to analyse whether treatment of HA EA MSCs with HAeSP marrow conditioned medium would increase their chondrogenic ability as it does with the PeSP MSCs.

It was also demonstrated in Figure 4.10 that the chondrogenic ability of MSCs is severely decreased when they are deprived of the initial days with bone marrow present. These results are consistent with certain results in previous studies. Bone marrow contains platelets and consequently is a rich source of growth factors, including PDGF and TGF-β (McCarrel et al., 2009). These growth factors are secreted by the MSCs (Mehtu et al., 2008) and have been shown to induce
chondrogenesis of MSCs themselves (Huang et al., 2008, Indrawattana et al., 2004). Therefore the increase in chondrogenesis may be due to the HA activating the cells present in suspension in bone marrow.

The loss of endogenous HA in OA joints perpetuates degradation of articular cartilage. The phenomenon seen in this study may be mimicking the micro-environment of in vivo cartilage which may encourage the cells present to secret more chondrogenic inducing factors due to the addition of the HA-marrow-conditioned medium. It is well-known that a fundamental characteristic of aging is that the body has a reduced capacity to regenerate damaged or diseased tissues. This reduced regenerative ability is partially due to age-related changes in the niche (Morrison and Spradling, 2008). This means that the addition of HA into the aging joint micro-environment could re-activate the protective action of HA or the exogenous addition of HA-conditioned autologous cells could increase regeneration of damaged cartilage. As reviewed by Chen, there are critical factors present in the marrow micro-environment that are responsible for the maintenance of MSC properties or for their stemness which is missing in our standard culture systems (Chen, 2010). The niche controls dormancy, self-renewal, progenitor output and early lineage decisions. It appears that addition of exogenous HA has a role in influencing these decisions and could be used where endogenous HA has been degraded in disease states.

As demonstrated in Figure 4.8, it appears that it is not necessary for bone marrow to be present for long periods for osteogenic differentiation as there was significantly higher osteogenic differentiation in the MSCs that had the bone marrow removed before sub-plating after 24 hrs. This adds more evidence to the hypothesis that the non-adherent population contains osteogenic precursors (Leonardi et al., 2009; Wlodarski and Wlodarski, 2004; Long et al., 1990). Because these cells are already osteogenic, they may not require the support of the bone marrow niche to differentiate and thrive. This is further backed up by the fact that there are no differences in the adipogenic differentiation of the MSCs as demonstrated in Figure 4.7. Even those cells which are not supported by the bone
marrow do not have a decreased ability to differentiate. Taken together, these osteo- and adipo-genic results further reiterate the observation that the HA effect is a chondro-specific phenomenon.

We observed that the HAeSPs required the presence of bone marrow for an extended period of time in primary culture to positively react to the HA and to activate chondrogenic pathways. Based on that observation alone, one could argue that the HA EA (early adherent) were less chondrogenically active for the reason that the bone marrow was washed off at the early time-point of 24 hours (Figure 2.9). However, there was also the HA LA (late adherent) populations which were even less chondrogenic than the HA early adherent MSCs (Figure 2.9) and they were in the presence of BM for 5 days. Thus the conclusion can be made that non-adherent bone marrow cells are a rich source of chondroprogenitors when specifically activated by HA.

In OA, HA synthesis is disrupted by the presence of pro-inflammatory cytokines, proteinases and free-radicals in the disease state. When HA is disrupted, it cannot maintain the mechanical integrity of the joint (Goldberg and Goldberg, 2010). As discussed in section 1.7.2, HA has been injected into OA joints resulting in therapeutic benefits. As HA is such an integral part of the knee’s microenvironment, cartilage and overall function, it may be that this relatively short exposure of injected HA activates the resident stem cells to exert a reparative or pain relieving effect. As outlined by Shen et al, the interaction of HA with cell surface receptors activates protein tyrosine and serine/threonine kinases such as Src, HER2/Neu receptor, focal adhesion kinase, MAP kinases and protein kinase C. It is believed that interactions with specific HA receptors selectively couple with their downstream signalling pathways; consequently, HA promotes the expression of specific cytokines and proteins involved in ECM remodelling (Turley et al., 2002). Another important function of HA is to immobilize proteoglycan in the cartilage. CD44-HA interaction is necessary for organisation, retention and regulation of the normal cartilage matrix. These physiological processes of endogenous HA along with its chondroprotective action in chondrocytes have also been demonstrated by
exogenously adding varying forms of HA (Goodstone et al., 2004; Kawasaki et al., 1999; Akmal et al., 2005; Grishko et al., 2009).

As lineage-directed differentiation of MSCs requires a specific micro-environment involving cells, signal molecules and scaffolds/carriers, it appears in this study that the HA present in the marrow is an excellent environment to induce the stem cells present towards chondrogenesis. Addition of exogenous HA has a role in influencing these decisions and could be used where endogenous HA has been degraded in disease states.

As pointed out in the chapter 3 discussion, along with isolating an extremely effective chondrogenic cell, this study also points to the benefits of enhancing the numbers of MSCs by retrieving and re-plating the non-adherent cell population of bone marrow MSC culture. This enhancement in cell yield is achieved without functional disadvantages occurring to the cells compared to the typical MSCs isolated in labs for varying research purposes.

In the area of orthopaedic medicine there are many examples of applications involving local delivery of BM MSCs. These include spine fusion (Muschler et al., 2003), the repair of segmental bone defects (Quarto et al., 2001; Arinzeh et al., 2003) and repair of defects in articular cartilage (Wakitani et al., 2002; Wakitani et al., 2004; Kuroda et al., 2007; Ponticiello, Schinagl, Kadiyala, & Barry, 2000). Most of these studies use MSCs that have been expanded and passaged multiple times in reparative surgeries, for example to treat a patient’s full-thickness articular cartilage defect in the femoral condyle, Kuroda et al. used autologous bone marrow stem cells which once isolated, were expanded for four weeks before being re-implanted in surgery. Currently there is an extended culture period following tissue isolation and re-implantation and with this comes the added risk of contamination or alterations occurring in the cells. As we have shown in this thesis, a relatively short exposure of the marrow to HA results in the isolation of MSCs that can be activated effectively towards chondrogenesis. The novel time-frame employed and the collection of non-adherent sub-populations will also allow the yield and
reparative ability of BM-derived MSCs to be greatly increased with associated possibilities of earlier clinical delivery.
Chapter 5

Thesis Overview
5.1 Overview

This study has not only elucidated a process enabling the isolation of a highly chondrogenic population of cells from human bone marrow but also a process that enables the retrieval of a higher yield of MSCs than is typically isolated from bone marrow using traditional plastic-adherence methods.

Typically orthopaedic surgeons have used artificial metallic implants which involve invasive operations and usually require replacement again later on in life (Long et al., 1998; Fortin et al., 2002; Baoqin, 2006). More recently orthopaedists have been moving towards biologic and cellular therapies to treat patients. These treatments help improve the efficiency of traditional tools and reduce the need for invasive surgeries (Coccia, 2012). Cell transplantation trials in the clinic have shown modest results at best. Limitations include poor cell persistence, post-transplantation viability, cell relocation to other sites (Suuronen et al., 2008; Kuraitis et al., 2012), donor site morbidity, repair cell de-differentiation with expansion in vitro (Benya et al., 1978; Diaz-Romero et al., 2005), restricted cellular life span upon implantation (Brittberg et al., 2003; Guerit et al., 2012), spontaneous osteonecrosis after ACI treatment (Von Keudell et al., 2011) and increased treatment failure in subchondral bone defects that have had prior treatment before ACI (Minas et al., 2009). Another issue is the poor differentiation potential of OA derived chondrocytes (Tallheden et al., 2005). In the past decade adult stem cells have emerged as promising therapeutic agents for treatment of damaged tissues.

Stem cells are not isolated populations in vivo; they reside within tissue-specific niches which influence their functions and behaviour (Nurcombe et al., 2007). Suuronen et al. demonstrated an improvement in regenerative effects in skeletal muscle upon the non-invasive delivery of stem cells within an injectable matrix which was composed of muscular ECM components (Suuronen et al., 2006; Kuraitis et al., 2011). The work described in this thesis has reiterated what was shown in these previous studies, that the pre-conditioning of stem cells with ECM
components result in a better phenotype for regeneration, however, the previous studies were improved upon in this thesis by pre-conditioning whole bone marrow and its components with HA thus creating a highly potent MSC phenotype. HA has been used in many studies with MSCs and has proven to enhance cell proliferation and to promote production and deposition of matrix components, such as collagen I, collagen III, fibronectin, and laminin (Cristino et al., 2005; Pasquinelli et al., 2008). MSCs expanded ex vivo can display reduced adhesion and migration capabilities; this has been shown to be reversed by supplementing cultures with HA (Jung et al., 2011). MSC incorporation onto HA-based scaffolds is also known to induce chondrogenic differentiation and deposition of a cartilage-like ECM (Jakobsen et al., 2010; Chung et al., 2009). Additionally, endogenous HA has pro-survival effects on cells through the activation of the anti-apoptosis Akt pathway (Toole., 2004) and by protection from toxic insult (Ratliff et al., 2010).

Taken together these studies demonstrate the positive effects of using ECM and HA treatments on passaged stem cells. The experiments carried out in this thesis improve upon these studies by way of using effective ECM molecules in the isolation of MSCs directly from bone marrow. It was shown in this work that the changes that HA exposure exerts upon MSCs at isolation are retained throughout passaging, demonstrating that these induced properties are maintained long enough for successful therapeutic application. Most importantly chondrogenic differentiation of MSCs exposed to HA during the isolation process was significantly increased over traditionally isolated MSCs using these novel methods. Using our method of isolation, it was demonstrated that the expression of the key chondrogenic transcription factor SOX9, was 20-fold higher than that of the traditionally isolated MSCs. Furthermore, by adapting the process, it was also demonstrated by subsequent methods of isolating MSCs using HA and by using varied time-points, that final cell yield was increased. This method therefore significantly increases the number of MSCs available for isolation from a single 50 ml sample of human marrow. MSCs were exposed to HA using direct plating of BM onto adsorbed HA at day 1 and day 5, by using the non-adhered populations at day 1 and day 5 and by adding the HA directly into the bone marrow for 24 hours.
before plating and cell isolation. It was also demonstrated that plastic or traditionally isolated MSCs can be primed in a similar fashion, not by adding HA alone but by supplementation with HA and marrow conditioned medium. This thesis has shown that the relatively quick exposure of HA to the BM upon the isolation of MSCs, the novel time-frame employed and the collection of non-adherent sub-populations all allow the yield and reparative ability of BM-derived MSCs to be greatly increased.

It is also important to note that the cells present in the day 5 cultures (LA) and the cells remaining non-adhered in the day 5 cultures (ISP) are functional and useable in laboratories for further study or as potential cell therapies. In total, the isolation methods described resulted in the isolation of 20 various populations of fully functional MSCs; we chose to focus on the cells with the highest chondrogenic potential, and the earliest isolation time-point as their characteristics are ideal for clinical translation. These results provide major advantages for clinical OA therapies using MSCs.

The following sections will summarise the main findings of each chapter and their implications.

5.2 Summary of Main Findings of each Chapter and their Implications

5.2.1 Chapter 2: Elucidation of the most efficient ECM molecule for the isolation of a chondrogenic population of MSCs

It was hypothesised that there is a sub-population of progenitor cells in bone marrow that are primed towards the chondrogenic pathway with pre-requisite receptors for cartilage ECM molecules. Furthermore, these chondroprogenitors can be isolated from bone marrow via their specific adhesion to these cartilaginous ECM molecules. Following preliminary analysis of various cartilaginous ECM molecules, three different matrix molecules were selected; two types of hyaluronic
acid (HA 1 and HA 2), chondroitin-6-sulfate (CS) and heparan sulfate which were all compared to each other and traditionally isolated plastic adherent MSCs. Cell culture surfaces were coated with these cartilaginous matrix molecules and adherent populations were isolated after whole bone marrow plating. Two different time-points (EA and LA) were analysed to determine if there was a sub-population of cells that would attach to the HA or CS within the first 24 hours as opposed to those that were left for 5 days to adhere. Upon chondrogenic differentiation of the MSCs isolated on the ECM coatings, it was demonstrated that Durolane (HA 1) EA and CS EA isolated MSCs created significantly more GAG compared to the MSCs isolated on the other ECM molecules and those with the BM residing on the plate for 5 days. The following experiments with HA 1 (Durolane-now simply referred to as HA) and CS coated plates were then performed. With the evident differences in these cells’ differentiation potentials, it was important to assess whether the cells retained their typical MSC characteristics. Results demonstrated that MSCs isolated using ECM molecules at EA or LA time-points had the characteristic MSC cell surface receptors present. All populations were over 96% positive for standard MSC markers, cells were shown to have typical MSC population doublings, all populations retained MSC phenotype, colony forming ability and all were positive for tri-lineage differentiation. Taken together, these results enabled the matrix isolated cells to remain defined as MSCs. Following chondrogenic differentiation a significant increase was observed in HA EA and CS EA compared to the other populations. HA EA MSCs were also shown to have significantly higher levels of SOX9 than all other time-points and treatment groups. The experiment carried out to analyse whether CS and HA also had positive effects on traditionally isolated, passage 1 MSCs demonstrated no significant differences between the CS or HA treated and non-treated passage 1 MSCs. It appears that HA and CS have a chondrogenic enhancing effect only upon isolation. This may be due to progenitor cells with ECM specific receptors present in the marrow becoming concentrated and binding rapidly to the HA or CS coated to the plates. The early isolation of this population and subsequent removal of competing factors after 24 hours in culture may allow this chondrogenic population to thrive and proliferate ensuring a high number of chondroprogenitors in the population.
5.2.2 Chapter 3: Analysis of the non-adherent MSC population

The objectives in this chapter were to assess the EA and LA non-adherent populations for putative MSCs, to compare these cells to those isolated by traditional methods and the adherent ECM-isolated populations described in the previous chapter, and to compare these EA non-adherent and LA non-adherent populations (early sub-population and late sub-population) to all other aforementioned MSC populations for differentiation potential. More substantially, to assess whether the early non-adherent sub-population is depleted in chondroprogenitors due to the increased chondrogenic differentiation in the HA and CS EA MSC populations described in Chapter 2.

It was expected that there would be MSCs in the early sub-population as the cells were given only 1 day to adhere; however, it was not expected that there would be functional MSCs remaining non-adhered in the marrow that was plated for 5 days. These late sub-population cells (along with all other populations) were shown, through phenotypic and functional analysis to be fully functional in terms of colony forming ability, proliferation, MSC surface expression and differentiation.

It was also hypothesised that there would be a reduction in the amount of chondroprogenitor cells in the sub-populations of the HA and CS isolated MSCs. This is because there were significantly more chondrogenic MSCs present in the ECM-isolated adherent populations so the non-adherent populations should theoretically have less chondroprogenitors. The resultant 9-fold increase in HAeSP chondrogenic potential over traditionally isolated MSCs was therefore unforeseen. It consequently appeared that the cells do not require initial adherence to the HA adsorbed to the tissue culture flask to be selected or activated from the marrow. HA is very soluble and it is not unexpected that a portion of the HA bound to the flask would become solvent in the marrow once it was plated. This would enable attachment or interaction of the non-adhered cells with HA. As expected, the
adipogenic ability of the sub-populations was not significantly altered as compared to the EA and LA populations. The CFU-F analysis did not demonstrate significant decreases. This reiterates that there are many functional non-adherent cells still present in the bone marrow after 24 hours and 5 days. Wan et al also demonstrated MSC cultures derived from the non-adherent cell population of human bone marrow had very similar proliferation and differentiation rates to the primarily adherent populations (Wan et al., 2005). This is an important observation that has been made as it hugely increases potential yield and potency from marrow isolations.

Molecular analysis showed that the expression of SOX9 had a 20-fold increase in the HAeSP MSCs compared to the PeSP MSCs after 24 hours in chondrogenic culture. Taken together, the significant increases that were demonstrated in the late and early events reveals that a highly chondrogenic population is isolated from the HA-exposed early sub-population. This population is significantly more chondrogenically active than the HA EA population isolated in the previous chapter. The SOX9 expression in HA EA was 3-fold higher than the plastic control whereas the SOX9 expression of HAeSP was 20-fold higher than the plastic control, similarly the 3 week chondrogenic differentiation of HAeSP MSCs demonstrated 3-fold higher GAG production than the HA EA MSCs. This could lead to a new method of chondroprogenitor isolation with higher numbers of MSCs isolated at the same time. Beyond isolating an extremely effective chondrogenic cell, this study also points out the benefits of enhancing the numbers of MSCs by retrieving and replating the non-adherent cell population of bone marrow MSC culture. This enhancement in cell yield is carried out without functional decline or disadvantages occurring to the cells compared to the typical MSCs isolated in labs for varying research purposes. Additionally, within some of these populations are the highly potent chondroprogenitors with 9-fold higher GAG production and a 20-fold increase in SOX9 expression when cultured in chondrogenic conditions.
5.2.3 Chapter 4: Investigation for:

1. An alternative method of ECM exposure,

2. Presence of a synergistic relationship existing between the BM and HA that is required for the HA to have its selective or activating effect on chondroprogenitors

An observation made in the previous chapter was that the cells do not in fact need to adhere to the adsorbed HA. The cells which were exposed to HA for the first 24 hours but that did not adhere were highly chondrogenic, hence, our hypothesis was that it may not be necessary for the HA to be coated onto the flasks. It may suffice to simply add the HA directly to the whole BM, perhaps even before BM processing and MSC isolation. After a 24 hour HA exposure in unprocessed marrow, the chondrogenic differentiation of isolated cells was shown to be significantly increased, affirming that it is not necessary for the cells to attach to immobilized HA coated on a flask to be chondrogenically activated. A significant increase in SOX9 expression was demonstrated in the HA-exposed chondrogenic MSCs as compared to the non-exposed MSCs. Significantly higher chondrogenic ability, measured by the GAG:DNA ratio, was also demonstrated compared to the non-exposed MSCs. This was shown to be solely a chondrogenic phenomenon as the adipogenic and osteogenic differentiation were not positively affected. Thus, it appears that the HA re-directs these potent progenitors down the chondrogenic lineage upon its exposure to in vivo MSC in their marrow micro-environment.

Secondly, due to the results shown in chapter 2, our rationale was that HA exposure in culture does not affect the chondrogenic potential of passaged MSCs. Only when HA is present upon the isolation of MSCs from the bone marrow is there a significant increase in chondrogenic potential. Based on this rationale, the hypothesis was proposed that there is a synergistic relationship between HA and the marrow niche that enhances the effect of HA on MSCs in the marrow, thus contributing to the enhancement in chondrogenic potential. We sought to isolate
the MCSs in the manner described in chapter 2, but after 24 hours in culture, remove the HA conditioned BM and add it to the cells that were not exposed to HA within the first 24 hours in culture and analyse these cells for a subsequent increase in chondrogenic potential. Hence, in this marrow conditioned media experiment, the hypothesis was that the PeSP cells would have a higher chondrogenic potential when treated with HAeSP-marrow conditioned medium and that the HAeSP populations would be chondrogenically inhibited when treated with the PeSP-marrow conditioned medium. If this was the case, it would demonstrate that HA plus BM could in fact activate plastic isolated MSCs, unlike HA alone which cannot. PeSP cells demonstrated a significant increase in chondrogenesis upon the addition of HAeSP marrow conditioned medium (the PeSP+HAeSP supernatant population). The exact opposite was demonstrated when HA isolated MSCs were treated with marrow conditioned medium from the PeSP cells. This demonstrates that bone marrow is a key factor during the induction of chondroprogenitors using HA.

Taken together these results tell us that in a clinical setting, passaged cells may be less potent than those which are isolated and re-implanted immediately with addition of HA. As outlined above, this study has elucidated processes enabling the isolation of a highly chondrogenic population of cells from the bone marrow and a process of MSC isolation from marrow that enables the retrieval of a higher yield of cells than is typically isolated by using traditional methods. This enhancement in cell yield is carried out without functional differences or disadvantages occurring to the cells. These processes may prove to be useful in general MSC isolation protocols and perhaps more importantly, the significant increase in chondrogenic potential of the HA early sub-population (HAeSP) MSCs may be very useful in the treatment of chondral lesions present in osteoarthritic patients.
5.3 Advancing the State of the Art- Novel Isolation methods of MSCs

Described below are some of the main important and novel findings from the work carried out in this thesis. The schematic outlined in Figure 5.1 describes the processes used to isolate the different MSC populations from the bone marrow.

- Twenty MSC sub-populations were isolated
- All retained differentiation potential and the typical MSC characteristics
- Three were highly chondrogenic populations resulting in more cells available for laboratory/clinical use
- Two were more homogeneous osteogenic populations
- It was concluded that the marrow needs to be present for HA to have an activating effect
- A process of isolation incorporating minimal marrow manipulation by exposure was developed. This process is amenable to rapid translation leading to more immediate use in the clinic with less time and cost incurred.
Figure 5.1: Schematic diagram of the current state-of-the-art in BM MSC Isolation Methods. The schematic describes the current state-of-the-art in the isolation of highly chondrogenic populations of BM MSCs based on the use of Hyaluronan upon isolation. Generally, the cells of the BM are isolated by direct plating and the plastic adherent cells are expanded for further use. The various methods of applying HA in MSC isolation (HA-coating, sub-plating the HA exposed MSCs and direct conditioning of the HA before BM processing) all resulted in highly chondrogenic populations of MSCs through activation of the MSCs by HA within the marrow micro-environment. It was also discovered that HA marrow conditioned medium can chondrogenically activate traditionally isolated MSCs.
5.4 Future Plans

This thesis demonstrated a method of isolating highly potent chondroprogenitors from bone marrow. Therefore an in vivo assessment would be of interest in order to analyse whether the chondroprogenitors are as potent in the in vivo setting as they are in vitro and whether they can form a stable form of hyaline cartilage that could repair damaged tissue in disease or injury. The ECM-Isolated chondroprogenitors could be used in an OA model such as the DMM SCID mouse model (destabilization of the medial meniscus) and analysed for cartilage regeneration and repair. The SCID model would enable the human cells to be used in an animal model without having to carry out cross-over studies to murine MSCs. There have been however, studies carried out recently using hMSCs in rat and mouse models (Horie et al., 2012; Liu et al., 2011) leaving cross-over studies less of a necessity.

For tissue engineering purpose, it would be worthwhile to combine the chondroprogenitors with a cartilage promoting scaffold to enable the differentiation process. There have been studies carried out using biomimetic scaffolds such as collagen/GAG scaffolds (Matsiko et al., 2012). These biomimetic scaffolds using HA and CS may provide ideal scaffolds to house the chondroprogenitors while in vivo. Also, if this protocol was to be carried forward to be used clinically, it would be necessary to develop a GLP SOP for HA isolated cell production.
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